PCT/DK 2004/000282

Kongeriget Danmark

Patent application No.: PA 2003 00616

Date of filing: 23 April 2003

Applicant: (Name and address)
Borean Pharma A/S
Gustav Wieds Vej 10
DK-8000 Aarhus
Denmark

Title: Cleavage of fusion proteins using Granzyme B protease

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed apart from page 1 which is filed 24 April 2003.

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

18 May 2004

Mette B. Nielsen

PATENT- OG VAREMÆRKESTYRELSEN

CLEAVAGE OF FUSION PROTEINS USING GRANZYME B PROTEASE

Field of the invention

The present invention relates to a method for the preparation of a polypeptide of interest in authentic form by enzymatic cleavage of recombinantly produced fusion proteins by the use of Granzyme B protease. Furthermore, the invention pertains to fusion proteins comprising a Granzyme B cleavage site.

Background of the invention and prior art

The production and purification of recombinant polypeptides such as pharmaceutical proteins in a highly purified and well-characterized form, has become a major task within the area of protein engineering in general, and in the pharmaceutical industry in particular.

The preparation of such recombinant polypeptides relies frequently on techniques which involve the production of the polypeptides as fusion proteins or hybrid proteins, wherein a protein or polypeptide of interest is fused to a carrier or a fusion partner such as a polypeptide or protein.

The presence of a fusion partner or carrier which is fused to the polypeptide of interest has the advantages that it may render the fusion protein more resistant to proteolytic degradation, may facilitate enhanced expression and secretion, improve solubility and allow for subsequent affinity purification of the fusion protein. Also by fusion protein expression, potentially bio-hazardous materials, such as peptide hormones, may be produced in an inactive form which can then be activated subsequently *in vitro* by cleaving of the fusion partner.

However, such fusion proteins themselves are not normally suitable as end products as the fusion partner e.g. may affect the biological activity or stability

of the polypeptide of interest and, if the protein is to be used clinically, may cause antigenicity problems. Therefore it is necessary to cleave the fusion protein to release the polypeptide of interest.

In principle this can be achieved by chemical or biochemical methods such as enzymatic cleavage. However, it is important that the cleavage is highly specific and only takes place in a cleavage sequence between the protein of interest and the fusion partner, i.e. the junction region, but preferably not within the protein of interest itself, as this may e.g. severely affect the bioactivity of the protein of interest. Such methods employ agents that act by hydrolysis of peptide bonds and the specificity of the cleavage agent is determined by the identity of the amino acid residue at or near the peptide bond which is cleaved.

Biochemical methods for cleavage of fusion proteins are based on the use of proteases (proteolytic enzymes). However, enzymatic cleavage of fusion proteins is limited in that the amino acid(s) which are specific for the cleavage site can at the same time also occur in the protein of interest itself. Therefore, enzymes are particularly suitable which, in order to cleave, not only recognises one amino acid but rather a sequence of amino acids, since the probability that a particular amino acid sequence is present once again in the protein of interest in addition to the cleavage site between the protein of interest and the fusion partner is less the larger the number of amino acids necessary for the recognition and cleavage of the cleavage sequence.

Up till now, a number of proteases have been used for enzymatic cleavage of fusion proteins by contacting the fusion protein with a protease under appropriate conditions.

WO 03/010204 relates to a process for separating a protein of interest from a fusion protein by the use of ubiquitin cleavage enzyme, which according to this document is an enzyme that cleaves a peptide bond next to the amino acid sequence RGG at the C-terminus of proteins such as ubiquitin.

US 6,010,883 disclose a method wherein blood clothing factor Xa (EC 3.4.21.6; a S1 serine-type peptidase formed from the proenzyme factor X by limited proteolysis) is used for cleaving off a fusion partner from a fusion protein. This protease specifically cleaves after the amino acid sequence X-Y-Gly-Arg, wherein X is Ile, Leu, Pro or Ala, and Y is Glu, Asp, Gln or Asn. Factor Xa preferably cuts after the cleavage sequence Ile-Glu-Gly-Arg.

Other prior art enzymes which have been suggested and used in methods for specific cleavage of fusion proteins include tobacco etch virus Nla proteinase, collagenase, enterokinase, subtilisin and thrombin.

However, several problems may be encountered when using proteolytic cleavage in fusion protein systems. One major problem is the occurrence of non-specific proteolytic attack of the fusion protein which results in cleavage at several locations and consequently product loss and generation of contaminating fragments. Also problems with inefficient or incomplete cleavage of the fusion protein frequently occur with the presently known enzymes. Such inefficient cleavage reduces the yield and may also introduce heterogeneity to the purified protein resulting in the recovery of only a small fraction of the desired protein.

A further problem that is associated with several of the presently applied enzymes for fusion protein cleavage is that spurious or extraneous amino acids are frequently attached to the cleaved polypeptide product (the polypeptide of interest). These amino acids are typically present when a linker is cleaved, and the unrelated amino acid residues may have an effect on the properties of the resulting protein of interest. This may be critical for proteins produced for human therapeutics. Therefore, it is highly desirable to be able to produce pure authentic polypeptides free of extraneous amino acid short sequences or residues.

The problem is illustrated in US 4,543,329 which describes a process for selectively cleaving a fusion protein by the use of collagenase. However, the use of this enzyme produces a protein of interest with the sequence Gly-Pro at

its N-terminal. In order to obtain the protein of interest in authentic form, these extraneous amino acids (Gly and Pro) must subsequently be removed in a further reaction step by the use of one or more different amino peptidases (such as aminoacylproline amino peptidase and proline amino peptidase).

The problem is also illustrated in US 5,427,927 which describe a process for sequence specific cleavage of fusion proteins by the use of IgA protease, wherein a IgA protease recognition site is inserted in the junction region of a fusion protein which is subsequently cleaved with IgA protease. The recognition site for the IgA protease is the amino acid sequence Y-Pro.!.X-Pro, in which X can be any amino acid, Y can be one or several arbitrary amino acids, and ! denotes the cleavage site. However, the proteins of interest which are formed after cleavage by IgA protease, are characterised by having an X-Pro extraneous amino sequence at its N-terminal, i.e. the resulting protein of interest is not in its native or authentic form.

Presently, the most widely used proteolytic enzymes for fusion protein cleavage are the serine proteases factor Xa and thrombin. However, both enzymes are known to perform non-specific cleavage of fusion proteins. In addition, factor Xa has to be isolated from bovine serum and as a consequence when it is used to cleave proteins for therapeutic applications an extensive purification and analysis is necessary afterwards in order to detect pathogenic factors such as viruses and prions which may be present (e.g. prions causing bovine spongiform encephalopathy). Furthermore, these enzymes are rather expensive.

In view of these prior art shortcomings and drawbacks, it is therefore an object of the present invention to provide an improved method for enzymatic cleavage of fusion proteins.

It has been found by the present inventors, that the above technical problems may be overcome by using Granzyme B protease (EC 3.4.21.79) for enzymatic cleavage of fusion proteins. Thus, it has surprisingly been found that Granzyme B protease allows for highly efficient cleavage of fusion proteins

having a Granzyme B protease cleavage site with a high degree of cleavage specificity. In particular Granzyme B protease has proven to perform more specific fusion protein cleavage than the presently and widely used protease factor Xa. It has furthermore been found that Granzyme B cleavage of fusion proteins that contain a Granzyme B recognition sequence positioned between an N-terminal fusion partner and a C-terminal polypeptide of interest, results in a polypeptide of interest that have no extraneous amino acids derived from the cleavage site, i.e. a polypeptide in authentic form. Thus, recombinant proteins of interest with native amino acid sequence may be produced as a result of fusion protein cleavage by Granzyme B. Finally, the Granzyme B protease has the advantage that it can be produced recombinantly.

Summary of the invention

Accordingly, the present invention relates in a first aspect to a method for the preparation of a polypeptide of interest in authentic form. The method comprises the steps of (i) providing a fusion protein having a polypeptide of interest which is fused to a fusion partner, where the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease recognition site having a Granzyme B protease cleavage site adjacent to the protein of interest, and (ii) contacting the fusion protein with Granzyme B protease (EC 3.4.21.79) to cleave it at the cleavage site to yield the protein of interest in authentic form.

In a further aspect there is provided a fusion protein comprising a polypeptide of interest and a fusion partner, wherein the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease recognition site having a Granzyme B protease cleavage site adjacent to the protein of interest.

In still further aspects there is provided an isolated nucleic acid sequence encoding such a fusion protein, a recombinant vector comprising the isolated nucleic acid sequence, a host cell transformed with such a vector, and a

method for the production of the fusion protein which comprises the steps of (i) providing such a recombinant vector which is operatively linked to a promotor, (ii) transforming a host cell with the recombinant vector, (iii) culturing the host cell under conditions to express the fusion protein, and (iv) optionally isolating the fusion protein.

Detailed disclosure of the invention

In one aspect the present invention relates to a method for preparing a polypeptide of interest in authentic form by enzymatic cleavage of fusion proteins. Accordingly, the method comprises, as is mentioned above, the steps of providing a fusion protein comprising a polypeptide of interest and a fusion partner wherein the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease recognition site which has a Granzyme B protease cleavage site adjacent to the polypeptide of interest. The fusion protein is subsequently contacted with Granzyme B protease to cleave the fusion protein at the Granzyme B protease cleavage site to yield the polypeptide of interest in authentic form.

In accordance with the present invention there is provided a method for producing polypeptides of interest in authentic form. As used herein, the term "authentic form" refers to a polypeptide which comprises the amino acid sequence thereof without any additional amino acid residues. As described above, a major problem associated with several of the presently applied enzymes for fusion protein cleavage is that spurious or extraneous amino acids frequently remains attached to the cleaved polypeptide product, i.e. resulting in a polypeptide which is not in an "authentic form". Thus, in the present context the polypeptide of interest in authentic form refers to a polypeptide having the same primary amino acid sequence as that encoded by the native gene sequence coding for the polypeptide of interest, i.e. it does not contain any non-native amino acids. It will be appreciated that a polypeptide of interest in authentic form not necessarily is a polypeptide that occurs in nature, but it may also be partially or completely artificial. In contrast, a "non-authentic"

polypeptide contains at least one amino acid which is not encoded for by the native gene sequence coding for the polypeptide of interest.

In accordance with the invention, the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease recognition site which has a Granzyme B protease cleavage site. Such a recognition site refers to a defined amino acid sequence that allows Granzyme B to recognize and to cleave the junction region between the protein of interest and the fusion partner. This junction region may be of any suitable length. Thus, it is contemplated that the junction region may in useful embodiments be in the form of a linker sequence. However, in order to obtain the protein of interest in authentic form, it is preferred that the Granzyme B cleavage site is positioned adjacent to the protein of interest in order to allow for specific cleavage of the fusion protein without resulting in spurious or extraneous amino acids remaining attached to the resulting protein of interest. Hence, the term "adjacent to" imply that the Granzyme B recognition sequence, which in some embodiments may be preceded by a linker sequence, is positioned such that the Granzyme B cleavage site is flanking the N-terminal of the polypeptide of interest.

Granzymes are granule-stored serine proteases that are implicated in T cell and natural killer cell-mediated cytotoxic defence reactions after target cell recognition. The principal function of granzymes is to induce the death of virus-infected and other potentially harmful cells. Granzyme B is one type of granzymes, and upon target cell contact it is directionally exocytosed and enters target cells assisted by perforin (a cytolytic protein expressed by cytotoxic T cells and natural killer cells). Granzyme B processes and activates various pro-caspases, thereby inducing apoptosis in the target cell. In accordance with the invention, the term "Granzyme B protease" includes enzymes which are classified under the Enzyme Commission number EC 3.4.21.79 in Enzyme nomenclature database, release 30, March 2003 (http://www.expasy.org/enzyme). Thus, in accordance with the invention any suitable Granzyme B protease may be used including human Granzyme B protease, mouse Granzyme B protease and rat Granzyme B protease. It is

generally preferred to use human Granzyme B, when the method in accordance with invention is used for the preparation of human therapeutic protein products. Human Granzyme B protease occurs in most human tissues where its biological function is fairly well known. Therefore, the presence of trace amounts of residual Granzyme B protease in the final therapeutic protein product exhibit a minimal risk for the patient to whom the therapeutic protein product is administered. Thus, it is known that if active Granzyme B protease is injected into the human blood stream it is swiftly trapped by alpha-2-macroglobulin and the complex is cleared via the LRP scavenging receptor. Granzyme B protease is also known under the alternative name "Cytotoxic t-lymphocyte proteinase 2".

Granzyme B protease is known to have a preference for cleaving after aspartate residues (D), and Granzyme B is the only mammalian serine protease known to have this P1-proteolytic specificity. Hence, it is contemplated that the Granzyme B cleavage site in useful embodiments at least comprises an aspartate residue at the P1 position located N-terminally to the cleavage site. Some of the presently known Granzyme B protease recognition sites are disclosed in Harris et al (1998). Thus, in useful embodiments, the recognition site has an amino acid sequence of the general formula: P4 P3 P2 P1↓ located N-terminally to cleavage site, wherein P4 preferably is amino acid I or V, P3 preferably is amino acid E, Q or M, P2 is X, where X denotes any amino acid, P1 preferably is amino acid D, and ↓ is the cleavage site for the Granzyme B protease.

It was found by the present inventors, that Granzyme B protease is capable of cleaving off polypeptides from a fusion protein, without leaving any non-native amino acids on the polypeptide. In particular it was surprisingly found that Granzyme B would recognise and cleave off polypeptides from a fusion protein after the P1 position without any strict requirements for specific amino acid residues at the P1'-P4' positions, i.e. the amino acid positions following the cleavage site. This is contrary to the findings in the prior art. In e.g. Sun et al. (2001) it is concluded that the P1'-P4' residues of Granzyme B substrates are

important for substrate binding, and that highest affinity for the substrate is observed when an acidic P4' residue is present (i.e. either aspartate or glutamic acid). Furthermore, Harris et al. (1998) concluded that Granzyme B has a strong preference for glycine residue at the P2' position. Despite these prior art findings, it has now been established that Granzyme B protease may be generally used for cleaving off polypeptides of interest from fusion proteins, without the need for specific amino acid residues at the P1'-P4' positions.

As mentioned above, a further particular advantage of the present invention is the finding that Granzyme B protease allows for highly efficient cleavage of fusion proteins having a Granzyme B protease cleavage site with a high degree of cleavage specificity. In particular it has been found that Granzyme B protease perform more specific fusion protein cleavage than the presently and widely used protease for fusion protein cleavage, namely factor Xa. Thus, as will be apparent from the below examples, it was found that when five different fusion proteins previously made as factor Xa cleavable fusion proteins, were cleaved by Granzyme B protease, it resulted in a cleavage performance that was as specific as or even more specific than that found with factor Xa. This may e.g. be seen from Example 5 and the accompanying Figure 9 illustrating an extended time course PAGE analysis of the digestion of the fusion proteins H6-FX-RAP and H6-GrB-RAP with factor Xa and Granzyme B, respectively. It is clearly seen from this experiment that there was an essentially complete cleavage after 30 min. of the fusion proteins with both proteases. However, more breakdown products were produced by the use of factor Xa as compared to Granzyme B. This clearly shows that Granzyme B is highly specific, and even more specific than the widely used protease factor Xa.

Although not necessary, it may in certain embodiments be advantageous to select the polypeptide of interest such that the polypeptide of interest, when it is a part of the fusion protein, N-terminally comprises the amino acids P1' and P2' resulting in the general Granzyme B recognition site formula P4 P3 P2 P1↓P1'P2' wherein P1' is X, where X denotes any amino, and P2' is G. Although Granzyme B protease has no strict amino acid selectivity for the P1'

position, there is a general preference for large hydrophobic amino acids at this position including Trp (T), Leu (L), Phe (F) and Ile (I). Thus, in one useful embodiment the amino acid at position P1' is selected from T, L, F and I. It may in a further aspect of the invention be advantageous that the polypeptide of interest is selected such that it, when being part of the fusion protein, N-terminally comprises an acidic amino acid at the P4' position, such as D or E.

In the present context the terms "amino acid" and "amino acid residues" refer to all naturally occurring L-alpha-amino acids. This definition is meant to include norleucine, ornithine, and homocysteine. The amino acids are identified by either the three-letter or single-letter designations:

Asp, D: aspartic acid	Ile,I: isoleucine
Thr, T: threonine	Leu, L: leucine
Ser, S: serine	Tyr, Y: tyrosine
Glu, E: glutamic acid	Phe, F: phenylalanine
Pro, P: proline	His, H: histidine
Gly, G: glycine	Lys, K: lysine
Ala, A: alanine	Arg, R: arginine
Cys, C: cysteine	Trp, W: tryptophan
Val, V: valine	Gln, Q: glutamine
Met, M: methionine	Asn, N: asparagine
Nle, J: norleucine	Orn, O: ornithine
Hcy, U: homocysteine	Xxx, X: any L-alpha-amino acid.

In further useful embodiments, the Granzyme B protease recognition site has an amino acid sequence which is selected from ICPD↓, IEAD↓, IEPD↓, IETD↓, IQAD↓, ISAD↓, ISSD↓, ITPD↓, VAPD↓, VATD↓, VCTD↓, VDPD↓, VDSD↓, VEKD↓, VEQD↓, VGPD↓, VRPD↓, VTPD↓, LEED↓, LEID↓, LGND↓, LGPD↓, AQPD↓, DEVD↓, where ↓ is the cleavage site for Granzyme B. These recognition and cleavage sites have previously been described by Casciola-Rosen et al. (1999).

As used herein, the term "polypeptide" should not necessarily indicate a limit on the size of the desired peptide polymer. Thus, this term is to be interpreted in its broadest sense, and hence includes peptides on the order of up to 50 or so amino acids, including oligopeptides such as di-, tri-, tetra-, penta-, and hexa-peptides, polypeptides and proteins.

In accordance with the invention the terms "polypeptide of interest" or "desired polypeptide" refer to the polypeptide whose expression is desired within the fusion protein. As previously described, in the fusion protein the polypeptide of interest will be joined or fused with another protein or protein domain, the fusion partner, to allow for e.g. enhanced stability of the polypeptide of interest and ease of purification of the fusion protein. In useful embodiments the polypeptide of interest is a protein such as a secreted protein. Secreted proteins have various industrial applications, including as pharmaceuticals, and diagnostics. Most protein drugs available at present, such as thrombolytic agents, interferons, interleukins, erythropoietins, colony stimulating factors, and various other cytokines, are secreted proteins. In a presently preferred embodiment the polypeptide of interest is a polypeptide hormone such as a polypeptide hormone selected from somatotrophin, glucagon, insulin and interferon.

In a further aspect of the invention the polypeptide of interest is an enzyme, such as Granzyme B. Thus, by providing a fusion protein in accordance with the invention and selecting Granzyme B protease as the polypeptide of interest, there is provided a self activating or autocatalytic Granzyme B protease which offers the possibility of providing inactive pro-Granzyme B which subsequently may be activated, in principle, by the addition of a single molecule of active Granzyme B protease. Thereby, there is provided pro-Granzyme B which is not dependent on the addition of e.g. external activator biologicals for its activation. As will be apparent from the following examples, the Granzyme B self activation was found to progress quantitatively to completion, and self activating samples of Granzyme B protease subjected to further incubation for several days were found to retain stable activity levels and produce minimal amounts of autolysis products. This clearly demonstrates

that self activating Granzyme B protease has the advantage of being highly stable to autolysis (cannibalism).

The fusion partner may, in accordance with the invention, be of any suitable kind provided that it is a peptide, oligopeptide, polypeptide or protein, including a di-peptide, a tri-peptide, a tetra-peptide, penta-peptide and a hexa-peptide. The fusion partner may in certain instances be a single amino acid. It may be selected such that it renders the fusion protein more resistant to proteolytic degradation, facilitate enhanced expression and secretion of the fusion protein, improve solubility, and allow for subsequent affinity purification of the fusion protein.

The fusion protein of the present invention may in useful embodiments comprise a fusion partner which is an affinity-tag. Such an affinity-tag may e.g. be an affinity domain which permits the purification of the fusion protein on an affinity resin. The affinity-tag may also be a polyhistidine-tag including hexahis-tag, a polyarginine-tag, a FLAG-tag, a Strep-tag, a c-myc-tag, a S-tag, a calmodulin-binding peptide, a cellulose-binding peptide, a chitin-binding domain, a glutathione S-transferase-tag, or a maltose binding protein.

In general the fusion partner will typically be selected on the basis of characteristics contributing to ease isolation, most desirable being those that are readily secreted by the microorganisms producing the fusion protein. Polyhistidine sequences, glutathione S-transferase and maltose binding protein, for example, are generally preferred as there are readily available affinity columns to which they can be bound and eluted from.

The method according to the invention may in useful embodiments include an isolation step for isolating the protein of interest which is formed by the enzymatic cleavage of the fusion protein, which has e.g. been immobilised by the use of the above mentioned affinity-tag systems. This isolation step can be performed by any suitable means known in the art for protein isolation, including the use of ion exchange and fractionation by size, the choice of which depending on the character of the polypeptide of interest.

In accordance with the invention the fusion protein is contacted with Granzyme B protease to cleave the fusion protein at the Granzyme B protease cleavage site to yield the polypeptide of interest in authentic form. This reaction may be carried out batchwise using free Granzyme B, or it may be carried out by using Granzyme B protease in an immobilised form, e.g. via adsorption, covalent binding, entrapment or membrane confinement. Suitable carriers for immobilised Granzyme B protease include conventional carriers such as polyacrylamide, chitin, dextran, kappa carrageenan, celite and cellulose. Immobilisation of enzymes by their covalent coupling to insoluble matrices is an extensively used technique. Lysine residues are found to be the most generally useful groups for covalent bonding of enzymes to insoluble supports due to their widespread surface exposure and high reactivity. Thus, in useful embodiments the Granzyme B protease is immobilised via a lysine amino acid residue. In a further aspect the Granzyme B protease is immobilised via its C-terminus, e.g. by means of a polyhistidine-tag, including a hexa-histidine-tag. The reaction may also be conducted by using a free Granzyme B protease in combination with a membrane-type bioreactor, or using a continuous type bioreactor together with an immobilised Granzyme B protease.

As will be apparent from the following examples, it has surprisingly been found that the time required for the cleavage of free fusion proteins (i.e. not immobilised) which comprises a polyhistidine fusion partner such as hexa-his, may be decreased dramatically if the fusion protein is contacted with Granzyme B protease in the presence of Ni^{2+} ions and Nitrilotriacetic Acid (NTA). It is contemplated that the main reason for this remarkable increase in cleavage speed, is that the Ni^{2+} ions bind the N-terminal polyhistidine fusion partner of the fusion protein and facilitate access for the Granzyme B protease to the cleavage site. Additionally it is also considered that the addition of NTA will shield the Ni^{2+} ions in solution in a similar fashion as on a Ni^{2+}-NTA agarose column, and thereby avoid precipitation of the resulting protein. When performing such a cleavage process, it is generally preferred that the concentration of Ni^{2+} is in the range of 1-20 mM, and the concentration of NTA is in the range of 1-20 mM. Furthermore, the temperature is preferably in the

range of 20-50°C, including the range of 25-45°C. In a preferred embodiment, the temperature is in the range of 35-40°C, such as about 37°C. In an even more preferred embodiment, the temperature is in the range of 40-45°C, such as about 42°C.

In accordance with the invention, there is also provided a fusion protein comprising a polypeptide of interest and a fusion partner, wherein the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease recognition site having a Granzyme B protease cleavage site adjacent to the protein of interest.

The fusion protein of the present invention may be expressed in any suitable standard protein expression system by culturing a host transformed with a vector encoding the fusion protein under such conditions that the fusion protein is expressed. Preferably, the expression system is a system from which the desired fusion protein may readily be isolated and refolded *in vitro*. As a general matter, prokaryotic expression systems are preferred since high yields of protein can be obtained and efficient purification and refolding strategies are available. However, numerous host cells may be selected as appropriate for transformation and expression of the described fusion protein, including mammalian insect, fungal and bacterial host cells which are particularly desirable. Commonly used bacterial strains include Bacillus and Escherichia, including *E. coli*. Thus, it is well within the abilities and discretion of the skilled artisan, without undue experimentation, to choose an appropriate or favourite host and expression system. Similarly, once the primary amino acid sequence for the fusion protein of the present invention is chosen, one of ordinary skill in the art can easily design appropriate recombinant nucleic acid sequence or DNA constructs encoding the fusion proteins of the invention, taking into consideration such factors as codon biases in the chosen host, the need for secretion signal sequences in the host, the introduction of proteinase cleavage sites within the signal sequence, and the like. These recombinant DNA constructs may be inserted in-frame into any of a number of expression vectors appropriate to the chosen host. The choice of an appropriate or favourite

expression vector is, again, a matter well within the ability and discretion of the skilled practitioner. Preferably, the expression vector will include a strong promoter to drive expression of the recombinant constructs.

Finally, there is provided a method for the production of a fusion protein according to invention which comprises the steps of (i) providing a recombinant vector comprising the isolated nucleic acid sequence encoding the fusion protein of the invention according which is operatively linked to a promotor, (ii) transforming a host cell with this recombinant vector, (iii) culturing the host cell under conditions to express the fusion protein, and
(iv) optionally isolating the fusion protein.

The invention will now be described by way of illustration in the following non-limiting examples and figures.

Description of the figures

Figure 1 shows the activity of an incubation of GrB-H6 with FX_a followed for several days using the following colorimetric assay: 500 µl buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0), 4 µl 100 mM Ac-IEPD-*p*NA and 5 µl GrB-H6. A mixture of 100 µl GrB-H6 (approximately 10 µg) with 1 µl FX_a (1 mg/ml) was kept at 4°C during the incubation, and the activity was measured after 0 hours, 2 hours, 5 hours, 19 hours, 2 days and 5 days.

Figure 2 shows the SDS PAGE of samples from the H6-IEPD-TripUB and H6-IEPD-TN123 incubations after 12 hours incubation with GrB-H6.
Description of the lanes A-J:
A: Molecular weight marker
B: H6-IEPD-TripUB alone after 12 hours incubation
C: 200 µl H6-IEPD-TripUB + 1 µl GrB-H6 after 12 hours incubation
D: 200 µl H6-IEPD-TripUB + 10 µl GrB-H6 after 12 hours incubation
E: H6-FX-TripUB incubated with FX_a

F: H6-IEPD-TN123 alone after 12 hours incubation
G: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 12 hours incubation
H: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 12 hours incubation
I: GrB-H6 alone in the same concentration as in lanes D and H
J: Murine H6-FX-TN123 cleaved with FX_a
Lane B shows non-cleaved H6-IEPD-TripUB (1), where no GrB-H6 was added, while lanes C and D show the two incubations with 1 and 10 µl GrB-H6 added. In both these lanes the product of the cleavage reaction; correctly cleaved H6-IEPD-TripUB (2) is seen in addition to the non-cleaved fusion protein. In lane E the construct H6-FX-TripUB, containing the FX_a recognition site IQGR in place of the GrB recognition site IEPD, is cleaved by FX_a giving a product of the same size as the GrB-H6 cleaved H6-IEPD-TripUB.
Lanes F-J show the GrB-H6 + H6-IEPD-TN123 incubations after 12 hours. In lane F is shown non-cleaved H6-IEPD-TN123 (3). Lanes G and H show how H6-IEPD-TN123 is cleaved by GrB-H6 when no Ca^{2+} is present (4). The band pattern is explained in figure 12. In lane J the murine H6-FX-TN123 construct has been cleaved by FX_a showing the size of the correctly cleaved product. Marked by (5) in the figure is the position of GrB-H6 with the same concentration as in the samples with 10 µl GrB-H6 added.

Figure 3 shows the SDS PAGE of the samples from the GrB-H6 + H6-IEPD-TripUB incubations after 12, 19 and 24 hours of incubation, as well as the samples from the GrB-H6 + H6-IEPD-TN123 incubations.
Description of the lanes A-K:
A: Molecular weight marker
B: H6-IEPD-TripUB alone after 12 hours incubation
C: 200 µl H6-IEPD-TripUB + 1 µl GrB-H6 after 12 hours incubation
D: 200 µl H6-IEPD-TripUB + 1 µl GrB-H6 after 19 hours incubation
E: 200 µl H6-IEPD-TripUB + 1 µl GrB-H6 after 24 hours incubation
F: 200 µl H6-IEPD-TripUB + 10 µl GrB-H6 after 19 hours incubation
G: 200 µl H6-IEPD-TripUB + 10 µl GrB-H6 after 24 hours incubation
H: GrB-H6 alone diluted as in F and G
I: H6-IEPD-TN123 alone after 12 hours incubation

J: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 12 hours incubation
K: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 12 hours incubation
Lane B shows non-cleaved H6-IEPD-TripUB (1). In lanes C-E the correctly cleaved product appears in all lanes, marked by (2) in the figure. The more GrB-H6 added and the longer the incubation time, the more cleavage product appears in the lanes.
Lanes I, J, and K in Figure 3 are identical to lanes F, G, and H in figure 2 with the H6-IEPD-TN123 + GrB-H6 incubations, though a larger sample has been run on the gel in Figure 3. The bands are therefore much clearer than in figure 2. The band marked with (3) is non-cleaved H6-IEPD-TN123 and the band pattern marked with (4), (5), (6) and (7) is explained in figure 12.

Figure 4 explains the simple band pattern observed in figures 2 and 3. When no GrB-H6 is added, no cleavage occurs and only the band from the non-cleaved fusion protein is seen in the gel. When GrB-H6 is added, the small N-terminal sequence is cleaved off and the correctly cleaved product appears on the gel in addition to the remaining non-cleaved fusion protein. The small N-terminal sequence cleaved off by GrB-H6 is too small to be visualized on the SDS gel.

Figure 5, 6 and 7 show the SDS PAGE of the samples from the H6-IEPD-TripUB + GrB-H6 incubations at 23°C (figure 5), 37°C (figure 6) and 42°C (figure 7) with no addition (1), addition of 4.2 mM Ni^{2+} (2) and addition of 4.2 mM Ni^{2+} + 5 mM NTA (3). Description of the lanes A-K (same for all temperatures):
A: Molecular weight marker
B: Non-cleaved H6-IEPD-TripUB
C: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, no addition
D: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, no addition
E: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, no addition
F: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+}
G: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+}
H: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+}

I: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+} and 5 mM NTA
J: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+} and 5 mM NTA
K: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6, 4.2 mM Ni^{2+} and 5 mM NTA
In lanes C-E (1) in all three figures where no Ni^{2+} or NTA was added, the H6-IEPD-TripUB fusion protein is cleaved at different rates for different temperatures. After 22 hours at 23°C approximately 40% of the fusion protein has been cleaved. At 37°C and 42°C more was cleaved after 22 hours than at 23°C, approximately 60% at 37°C and 50% at 42°C.
Because of the precipitation of protein observed at 37°C and 42°C with 4.2 mM Ni^{2+} no further cleavage of the fusion protein after 2 hours incubation was seen in the gel. Therefore less protein was seen in lanes F-H (2) in figures 6 (37°C) and 7 (42°C) than in lanes F-H (2) in figure 5 (23°C). In figure 5, lanes F-H (2), approximately 50 % of the fusion protein was cleaved to product after 22 hours incubation, which is more than was cleaved with no addition of Ni^{2+}.
No precipitation was observed with 4.2 mM Ni^{2+} + 5 mM NTA added to the incubations. In figure 5 lanes I-K (3) more product is seen than in lanes C-E (1) and F-H (2), so after 22 hours incubation at 23°C with both Ni^{2+} and NTA present approximately 60 % of the fusion protein has been cleaved compared to only about 40 % with no addition and 50 % with Ni^{2+} alone.
By further increasing the temperature to 37°C (figure 6 lanes I-K (3)) and 42°C (figure 7 lane I-K (3)) an even greater increase in the rate of cleavage is seen. After 22 hours of incubation at 37°C almost all the fusion protein is cleaved to the correct product. A little less is cleaved at 42°C after 22 hours.

Figure 8 shows the SDS PAGE of the samples from the H6-IEPD-RAP incubations with 1 or 10 µl GrB-H6. Description of the lanes (A-L):
A: Molecular weight marker
B: H6-IEPD-RAP alone after 5 hours incubation
C: 200 µl H6-IEPD-RAP + 1 µl GrB-H6 after 5 hours incubation
D: 200 µl H6-IEPD-RAP + 10 µl GrB-H6 after 5 hours incubation
E: H6-IEPD-RAP alone after 23 hours incubation
F: 200 µl H6-IEPD-RAP + 1 µl GrB-H6 after 23 hours incubation

G: 200 µl H6-IEPD-RAP + 10 µl GrB-H6 after 23 hours incubation
H: H6-FX-RAP cut partly with FX_a, purified
I: H6-FX-RAP cut almost completely with FX_a, purified
J: H6-IEPD-RAP alone after 26 hours incubation
K: 200 µl H6-IEPD-RAP + 1 µl GrB-H6 after 26 hours incubation
L: 200 µl H6-IEPD-RAP + 10 µl GrB-H6 after 26 hours incubation
Non-cleaved H6-IEPD-RAP (1) is shown in lanes B, E, and J. In lanes C and D it is clear that all the H6-IEPD-RAP has been cleaved to give the final product (2) after only 5 hours incubation with either 1 or 10 µl GrB-H6 as described above. It is also clear that there is at least one internal cleavage site in RAP giving rise to the two lower bands appearing in these lanes, i.e. the final product is cleaved into two pieces both visible on the gel (3). Lanes F and G and lanes K and L show essentially the same as lanes C and D, though the samples were taken later, after 23 and 26 hours of incubation with GrB-H6 giving rise to more cleavage in the apparent internal site in RAP.
In lanes H and I is shown purified samples of H6-FX-RAP cleaved partly (lane H) or completely (lane I) by FX_a to give the final RAP product. In these lanes the degradation products from any internal cleavage by FX_a has been removed by purification.

Figure 9 shows the SDS PAGE of the samples from the H6-IEPD-RAP + GrB-H6 and the H6-FX-RAP + FX_a incubations. Description of the lanes (A-O):
A: Molecular weight marker
B: H6-FX-RAP alone after 27 hours incubation
C: 400 µl H6-FX-RAP + 1 µl FX_a after ½ hour incubation
D: 400 µl H6-FX-RAP + 1 µl FX_a after 1 hour incubation
E: 400 µl H6-FX-RAP + 1 µl FX_a after 3 hours incubation
F: 400 µl H6-FX-RAP + 1 µl FX_a after 5 hours incubation
G: 400 µl H6-FX-RAP + 1 µl FX_a after 7 hours incubation
H: 400 µl H6-FX-RAP + 1 µl FX_a after 27 hours incubation
I: H6-GrB-RAP alone after 27 hours incubation
J: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after ½ hour incubation
K: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after 1 hour incubation

L: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after 3 hours incubation
M: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after 5 hours incubation
N: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after 7 hours incubation
O: 400 µl H6-GrB-RAP + 2 µl GrB-H6 after 27 hours incubation
In lanes B-H are the samples from the H6-FX-RAP incubation (1), where lane B shows non-cleaved H6-FX-RAP. Lane C-H shows that after only ½ hour almost all of the fusion protein has been cleaved by FX_a to give the correct product. In lanes D-G some degradation products show up, and in lane H after 27 hours of incubation all of the fusion protein has been degraded to give a variety of smaller pieces, and there is no correctly cleaved product left.
Lanes I-O shows the samples from the H6-GrB-RAP incubation (2). Lane I shows non-cleaved H6-GrB-RAP, and as for H6-FX-RAP nearly all the H6-GrB-RAP has been cleaved correctly after only ½ hour incubation with GrB-H6, as is seen in lane J. In lanes K-N degradation products show up, but not nearly as many as for the H6-FX-RAP incubation. In lane O after 27 hours of incubation there is still quite a lot of correctly cleaved product left.

Figure 10 shows the SDS PAGE of the samples from the H6-IEPD-TN123 + GrB-H6 incubation after 12 hours and 5 days without addition of Ca^{2+}. Some samples have been reduced. Description of the lanes (A-N):
A: Molecular weight marker
B: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 5 days incubation, sample reduced
C: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 5 days incubation, sample reduced
D + E: H6-IEPD-TN123 alone after 5 days incubation
F: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 5 days incubation
G: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 5 days incubation
H: GrB-H6 alone diluted as in C and G
I: H6-IEPD-TN123 alone after 12 hours incubation
J: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 12 hours incubation
K: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 12 hours incubation
L: H6-IEPD-TN123 alone after 12 hours incubation, sample reduced

M: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 after 12 hours incubation, sample reduced
N: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 after 12 hours incubation, sample reduced
Lanes I-K are identical to lanes F-H in figure 2 and I-K in figure 3, i.e. samples after 12 hours incubation with either 0, 0.2 or 2 µg GrB-H6. Here the band pattern **(1)** indicates an internal cleavage site in the TN123 part of H6-IEPD-TN123, and the pattern is further explained in figure 12. Lanes D-G show the incubations after 5 days, and here most of the fusion protein has been cleaved. In lane G (10 µl GrB-H6 added) almost all of the fusion protein has been cleaved twice **(2)**; at the IEPD↓ sequence as well as at the internal site in TN123 with the sequence AQPD↓.
Lanes L-N and lanes B-D show the same samples after 12 hours and after 5 days, respectively, but here the samples are reduced. This band pattern **(3)** is also explained in Figure 12 and again almost all of the fusion protein has been cleaved twice after 5 days with 10 µl GrB-H6, lane C **(4)**.

Figure 11 shows the SDS PAGE of the samples from the H6-IEPD-TN123 + GrB-H6 incubation after 12 hours and 2 days with the addition of 5 mM Ca^{2+}. Some samples have been reduced. Description of the lanes (A-K):
A: Molecular weight marker
B: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 and 5 mM $CaCl_2$, sample reduced
C: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 and 5 mM $CaCl_2$, sample reduced
D: H6-IEPD-TN123 alone
E: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 and 5 mM $CaCl_2$
F: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 and no $CaCl_2$
G: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 and 5 mM $CaCl_2$
H: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 and no $CaCl_2$
I: GrB-H6 alone diluted as in G and H
J: 200 µl H6-IEPD-TN123 + 1 µl GrB-H6 and no $CaCl_2$ after 2 days incubation
K: 200 µl H6-IEPD-TN123 + 10 µl GrB-H6 and no $CaCl_2$ after 2 days incubation

Lanes B-H and J-K show the incubations of H6-IEPD-TN123 with GrB-H6 after 12 hours and 2 days, respectively.
Lane D shows non-cleaved H6-IEPD-TN123. Comparing lane E and G (+ 5 mM Ca^{2+}) with lane F and H (no Ca^{2+}) only two bands appear with 5 mM Ca^{2+} present (1), while four bands appear (2) when no Ca^{2+} in present, as described for Figures 2, 3, 10 and 12. After 12 hours incubation with 10 µl GrB-H6 approximately 40 % of the fusion protein has been correctly cleaved when Ca^{2+} is present (lane G), while the cleavage of the two sites when no Ca^{2+} is present happens a bit faster (lane H after 12 hours and K after 2 days). In lane K almost all the fusion protein has been cleaved twice (3).
The samples in lanes B and C are reduced and still only two bands appear (4); the non-cleaved H6-IEPD-TN123 and the correctly cleaved product, where the H6 is removed.

Figure 12 shows a schematic representation of the band pattern observed on the SDS PAGE gels in figures 2, 3, 10 and 11.
(A): When no Ca^{2+} is present the H6-IEPD-TN123 construct is cleaved at two different sites indicated by "GrB-H6 →". The small N-terminal part cleaved off is too small to be visualized on the gel. The resulting molecule consists of two polypeptide chains held together by a disulfide bond.
(1) and (2): In a non-reducing gel the band pattern in (2) is obtained when the cleavage is not complete. (1) is the non-cleaved H6-IEPD-TN123, and in (2) the remaining non-cleaved fusion protein is the second band from the top. The top band in (2) is H6-IEPD-TN123 cleaved at the internal site, AQPD↓, giving a molecule of the same size as non-cleaved H6-IEPD-TN123, but less compact. The band at the bottom is the correctly cleaved fusion protein, whereas the third band from the top is the fusion protein cleaved twice; both at the correct IEPD↓ site and at the internal AQPD↓ site. When cleaved at the internal site the molecule is less compact and therefore migrates shorter in the gel than the correctly cleaved fusion protein.
(3) and (4): If the samples are reduced the band pattern in (4) is observed. Here any disulfide bonds are broken, so only single polypeptide chains are seen in the gel. (3) shows the position of the non-cleaved, reduced H6-IEPD-

TN123. The top band in (4) is the remaining non-cleaved H6-IEPD-TN123, while the second band from the top is the correctly cleaved and reduced H6-IEPD-TN123. Under the reducing conditions the molecules cleaved at the internal site are no longer held together by any disulfide bonds, and only the larger one of the two polypeptides after internal cleavage can be seen in the gel. Therefore the third band from the top is the larger part of the internally cleaved fusion protein, and the bottom band is this larger part after cleavage at both the internal site and at the correct IEPD↓ site.

(B): When 5 mM Ca^{2+} is added to the incubations, no internal cleavage is observed. Ca^{2+} ions bind to the H6-IEPD-TN123 molecule in a way preventing GrB-H6 from cleaving the fusion protein at the internal AQPD↓ site. With the AQPD↓ site rendered inaccessible cleavage only occurs at the correct IEPD↓ site.

(5) and **(6)**: When only cleavage at the correct IEPD↓ site occurs the band pattern in (6) is seen. The position of the non-cleaved H6-IEPD-TN123 is shown in (5), and so the top band in (6) is the remaining non-cleaved H6-IEPD-TN123. The bottom band is the fusion protein cleaved only once at the correct site. The small N-terminal peptide is too small to be visualized in the gel.

Figure 13 shows samples from the incubations of three of the five H6-TripUB variants with GrB-H6. The three variants are H6-IEPD-TripUB, H6-TripUB IQAD↓SP and H6-TripUB IQAD↓SG. Description of the lanes (A-P):

A: Molecular weight marker

B: H6-IEPD-TripUB alone after 24 hours incubation

C: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6 after 2 hours incubation

D: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6 after 6 hours incubation

E: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6 after 24 hours incubation

F: 200 µl H6-IEPD-TripUB + 5 µl GrB-H6 after 48 hours incubation

G: H6-TripUB IQAD↓SP alone after 24 hours incubation

H: 200 µl H6-TripUB IQAD↓SP + 5 µl GrB-H6 after 2 hours of incubation

I: 200 µl H6-TripUB IQAD↓SP + 5 µl GrB-H6 after 6 hours of incubation

J: 200 µl H6-TripUB IQAD↓SP + 5 µl GrB-H6 after 24 hours of incubation

K: 200 µl H6-TripUB IQAD↓SP + 5 µl GrB-H6 after 48 hours of incubation

L: H6-TripUB IQAD↓SG alone after 24 hours incubation
M: 200 µl H6-TripUB IQAD↓SG + 5 µl GrB-H6 after 2 hours incubation
N: 200 µl H6-TripUB IQAD↓SG + 5 µl GrB-H6 after 6 hours incubation
O: 200 µl H6-TripUB IQAD↓SG + 5 µl GrB-H6 after 24 hours incubation
P: 200 µl H6-TripUB IQAD↓SG + 5 µl GrB-H6 after 48 hours incubation
In lane B is shown non-cleaved H6-IEPD-TripUB, while more and more correctly cleaved product appear in lanes C-F after incubation with GrB-H6. In lane F after 48 hours incubation approximately 2/3 of the original amount of non-cleaved H6-IEPD-TripUB has been correctly cleaved. In lanes G-K is shown the H6-TripUB IQAD↓SP samples giving more or less the same picture as for H6-IEPD-TripUB with non-cleaved H6-TripUB IQAD↓SP in lane G and an increasing amount of correctly cleaved product in lanes H-K. The cleavage, though, is much slower than cleavage of the IEPD↓SP sequence and only a small amount has been cleaved after 48 hours incubation. For the H6-TripUB IQAD↓SG samples in lanes L-P it is evident that the cleavage is very much faster than for both H6-IEPD-TripUB and H6-TripUB IQAD↓SP. Lane L shows the non-cleaved H6-TripUB IQAD↓SG and already after only 2 hours incubation the majority of the fusion protein has been cleaved to give the correct product.

Figure 14 shows samples from the incubations of two of the five H6-TripUB variants with GrB-H6. The two remaining variants are H6-TripUB VGPD↓SP and H6-TripUB VGPD↓FG. Description of the lanes (A-K):
A: H6-TripUB VGPD↓SP alone after 24 hours incubation
B: 200 µl H6-TripUB VGPD↓SP + 5 µl GrB-H6 after 2 hours incubation
C: 200 µl H6-TripUB VGPD↓SP + 5 µl GrB-H6 after 6 hours incubation
D: 200 µl H6-TripUB VGPD↓SP + 5 µl GrB-H6 after 24 hours incubation
E: 200 µl H6-TripUB VGPD↓SP + 5 µl GrB-H6 after 48 hours incubation
F: H6-TripUB VGPD↓FG alone after 24 hours incubation
G: 200 µl H6-TripUB VGPD↓FG + 5 µl GrB-H6 after 2 hours incubation
H: 200 µl H6-TripUB VGPD↓FG + 5 µl GrB-H6 after 6 hours incubation
I: 200 µl H6-TripUB VGPD↓FG + 5 µl GrB-H6 after 24 hours incubation

J: 200 µl H6-TripUB VGPD↓FG + 5 µl GrB-H6 after 48 hours incubation
K: Molecular weight marker
In lane A is non-cleaved H6-TripUB VGPD↓SP and in lanes B-E more and more correctly cleaved product appears as seen for H6-IEPD-TripUB (lanes B-F figure 13). Approximately half the amount of fusion protein has been cleaved after 48 hours. In lane F non-cleaved H6-TripUB VGPD↓FG is shown and in lanes G-J the correctly cleaved product of H6-TripUB VGPD↓FG appears. After only 2 hours incubation all the fusion protein has been correctly cleaved.

Examples

Example 1

Design and construction of human Granzyme B expression vectors

A sequence encoding activated human Granzyme B (E.C. 3.4.21.79), i.e. from Ile21 (Ile16 in chymotrypsin numbering) to Tyr246, was cloned into pT7 cloning vectors containing a hexa-His tag (H6) either N- or C-terminally. A blood clotting factor X_a (FX_a) activation sequence of IEGR was placed just N-terminally to Ile21. A Granzyme B construct with the C-terminal hexa-His tag is shown in SEQ ID NO. 26. The expression vectors pT7-IEPD-GrB-H6 and pT7-IEAD-GrB-H6 were also constructed, wherein the FX_a activation sequence of IEGR was substituted with IEPD or IEAD, respectively. The design and cloning of the vectors is outlined in the following:

Construction of the pT7 C-term H6 cloning vector

The cloning vector pT7 C-term H6, was constructed by ligation of the DNA fragment made from the oligonucleotide primers H6 C-term fw (SEQ ID NO: 1) and H6 C-term rev (SEQ ID NO: 2) into an *Nco*I and *Eco*RI cut vector, pT7 (Christensen JH et al., 1991), using standard procedures.

Cloning of human Granzyme B into pT7 expression vectors

The expression vector pT7-GrB-H6, was constructed by ligation of the *Bam*HI and *Eco*RI restricted DNA fragment GrB EcoRI amplified from a mixture of cDNA, isolated from human bone marrow, human leukocyte, human lymphnodes, and lymphoma (Raji) cells (Clontech Laboratories, Inc cat # 7181-1, 7182-1, 7164-1, 7167-1) (with the oligonucleotide primers GrBfw (SEQ ID NO: 3) and GrBrev EcoRI (SEQ ID NO: 4)) into a *Bam*HI and *Eco*RI cut vector; pT7 C-term H6, using standard procedures. Outlines of the resulting nucleotide sequence of GrB EcoRI, is given as SEQ ID NO: 5.

The expression vector pT7-H6-GrB, was constructed by ligation of the *Bam*HI and *Hind*III restricted DNA fragment GrB HindIII amplified from a mixture of cDNA, isolated from human bone marrow, human leukocyte, human

lymphnodes, and lymphoma (Raji) cells (Clontech Laboratories, Inc cat # 7181-1, 7182-1, 7164-1, 7167-1) (with the oligonucleotide primers GrBfw (SEQ ID NO: 3) and GrBrev HindIII (SEQ ID NO: 6)) into a *Bam*HI and *Hind*III cut vector, pT7-H6 (Christensen JH et al., 1991), using standard procedures. Outlines of the resulting nucleotide sequence of GrB HindIII, is given as SEQ ID NO: 7.

Construction of expression vector for self-activating human Granzyme B

The expression vectors pT7-IEPD-GrB-H6 and pT7-IEAD-GrB-H6 were constructed by using the QuikChange™ Site-Directed Mutagenesis Kit (STRATAGENE, Catalog #200518) according to the manufacturers' protocol. The expression vector pT7-GrB-H6 was used as template. The oligonucleotide primers GrB GR-PD fw and GrB GR-PD rev (SEQ ID NO: 8 and 9) were used for construction of pT7-IEPD-GrB-H6 and the oligonucleotide primers GrB GR-AD fw and GrB GR-AD rev (SEQ ID NO: 10 and 11) were used for construction of pT7-IEAD-GrB-H6.

Table 1: Oligonucleotide primers

Primer	Nucleotide sequence	SEQ ID NO.
H6 C-term fw	5'-CATGGACGGAAGCTTGAATTCACATCACCATCACCATCACTA ACGC-3'	1
H6 C-term rev	5'-AATTGCGTTAGTGATGGTGATGGTGATGTGAATTCAAGCTTC CGCT-3'	2
GrBfw	5'-CATGGGATCCATCGAGGGTAGGATCATCGGGGGACATG AG-3'	3
GrBrev EcoRI	5'-GCGTGAATTCAGGTACCGTTTCATGGTTTTCTTTATCC-3'	4
GrBrev EcoRI	5'-GGCGAAGCTTAGTAGCGTTTCATGGTTTTC-3'	6
GrB GR-PD fw	5'-TCCATCGAGCCGGATATCATCGGGGGACATGAG-3'	8
GrB GR-PD rev	5'-CCCCGATGATATCCGGCTCGATGGATCCCATATG-3'	9
GrB GR-AD fw	5'-TCCATCGAGGCTGATATCATCGGGGGACATGAG-3'	10
GrB GR-AD rev	5'-CCCCGATGATATCAGCCTCGATGGATCCCATATG-3'	11

Example 2

Expression and refolding of the human Granzyme B

Recombinant human Granzyme B fusion protein GrB-H6 was produced by growing and expressing the vector pT7-GrB-H6 in *E. coli* BL21 cells in a medium scale (3x1 litre) as described by Studier FW et al. (1990). Exponentially growing cultures at 37°C were at OD_{600} = 0.8 infected with bacteriophage ?CE6 at a multiplicity of approximately 5. Cultures were grown at 37°C and 50 min after infection 0.1 g/L rifampicin (dissolved as 0.1 g/mL in methanol) was added. After another three hours at 37°C the cells were harvested by centrifugation. The cells were lysed by osmotic shock and sonification and total cellular protein was extracted into phenol (adjusted to pH 8 with Trisma base). The protein was precipitated from the phenol phase by addition of 2.5 volumes of ethanol and centrifugation. The protein pellet was dissolved in a buffer containing 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 100 mM dithiothreitol. Following gel-filtration on Sephadex™ G-25 Fine (Amersham Biosciences) into 8 M Urea, 0.5 M NaCl, 50 mM Tris-HCl pH 8, and 5 mM 2-mercaptoethanol, the crude protein preparation was applied onto a Ni^{2+}-activated NTA-agarose column (Ni^{2+}-NTA-agarose, Quiagen).

Upon application of the crude protein extract onto the Ni^{2+}-NTA -agarose column, the fusion protein, GrB-H6 was purified from the majority of *E. coli* and ? phage proteins by washing with one column volume of the loading buffer followed by one column volume of 8 M Urea, 0.5 M NaCl, 50 mM sodium phosphate pH 6.3 and 5 mM 2-mercaptoethanol, ½ column volume of 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 5 mM 2-mercaptoethanol and finally ½ column volume of 8 M Urea, 0.5 M NaCl, 50 mM Tris-HCl pH 8, and 3 mM reduced glutathione.

The GrB-H6 fusion protein was refolded on the Ni^{2+}-NTA -agarose column using the cyclic refolding procedure described by Thøgersen et al. (International Patent Application WO9418227). The gradient manager profile is described in Table 2 with 0.5 M NaCl, 50 mM Tris-HCl pH 8, 2 mM reduced

glutathione, and 0.2 mM oxidized glutathione as buffer A and 6 M urea, 0.5 NaCl, 50 mM Tris-HCl pH 8, and 3 mM reduced glutathione as buffer B.

After completion of the cyclic refolding procedure, the GrB-H6 fusion protein was eluted from the Ni^{2+}-NTA-agarose column with a buffer containing 0.5 M NaCl, 50 mM Tris-HCl pH 8, and 10 mM EDTA pH 8.

After elution from the Ni^{2+}-NTA column the GrB-H6 protein was diluted with 1 vol. of 50 mM Tris-HCl pH 8.0 before the pH was adjusted to 7 with HCl. The protein was then applied onto a SP Sepharose™ Fast Flow (Amersham Biosciences) ion exchange column. The protein was eluted over 10 column volumes with a linear gradient from 250 mM NaCl, 50 mM Tris-HCl pH 7.0 to 1 M NaCl, 50 mM Tris-HCl pH 7.0. Samples from the elution profile appear as a single distinct band in SDS-PAGE analysis and migrate with the anticipated molecular weight of 27.4 kDa for non-activated monomeric GrB-H6 (not shown).

Table 2:

Step	Time (min)	Flow (mL/min)	% A	% B
1	0	2	100	0
2	45	2	100	0
3	46	2	0	100
4	62	2	0	100
5	80	2	100	0
6	105	2	100	0
7	106	2	4	96
8	113	2	4	96
9	120	2	100	0
10	165	2	100	0
11	166	2	8	92
12	172	2	8	92
13	180	2	100	0
14	225	2	100	0
15	226	2	10	90
16	232	2	10	90
17	240	2	100	0
18	285	2	100	0
19	286	2	12	88
20	292	2	12	88
21	300	2	100	0
22	345	2	100	0
23	346	2	14	86
24	352	2	14	86
25	360	2	100	0
26	405	2	100	0
27	406	2	16	84
28	412	2	16	84
29	420	2	100	0
30	465	2	100	0
31	466	2	18	82
32	472	2	18	82
33	480	2	100	0
34	525	2	100	0
35	526	2	20	80
36	532	2	20	80
37	540	2	100	0
38	585	2	100	0
39	586	2	22	78
40	592	2	22	78
41	600	2	100	0
42	645	2	100	0
43	646	2	24	76
44	652	2	24	76
45	660	2	100	0
46	705	2	100	0
47	706	2	30	70
48	713	2	30	70
49	720	2	100	0
50	765	2	100	0
51	766	2	35	65
52	772	2	35	65
53	780	2	100	0
54	825	2	100	0
55	826	2	40	60
56	832	2	40	60
57	840	2	100	0
58	885	2	100	0
59	886	2	45	55
60	892	2	45	55
61	900	2	100	0
62	945	2	100	0
63	946	2	50	50
64	952	2	50	50
65	960	2	100	0
66	1005	2	100	0
67	1006	2	55	45
68	1012	2	55	45
69	1020	2	100	0
70	1065	2	100	0
71	1066	2	60	40
72	1072	2	60	40
73	1080	2	100	0
74	1125	2	100	0
75	1126	2	60	40
76	1132	2	60	40
77	1140	2	100	0
78	1185	2	100	0
79	1186	2	60	40
80	1192	2	60	40
81	1200	2	100	0
82	1245	2	100	0
83	1246	2	65	35
84	1252	2	65	35
85	1260	2	100	0
86	1305	2	100	0
87	1306	2	65	35
88	1312	2	65	35
89	1319	2	100	0
90	1364	2	100	0
91	1365	2	65	35
92	1371	2	65	35
93	1378	2	100	0
94	1423	2	100	0

Example 3

Activation of the GrB-H6 fusion protein using purified bovine Factor X_a and colorimetric activity measurements using the substrate Ac-IEPD-pNA

Activation of GrB-H6 by Factor Xa

A sample of monomeric GrB-H6 containing the IEGR sequence was taken directly from the eluate from the SP Sepharose ion exchange described in Example 2. 1 mg of GrB-H6 (in app. 10 ml) was activated by the addition of 50 µg FX_a (50 µl of 1 mg/ml) and incubated at RT for several days. The degree of cleavage/activation by FX_a was estimated by SDS PAGE (not shown).

To remove the added FX_a the activation mixture was loaded onto a SP Sepharose™ Fast Flow (Amersham Biosciences) ion exchange column washed in 250 mM NaCl, 50 mM Tris-HCl pH 7.0. The FX_a did not bind to the column material, while the activated GrB-H6 was eluted with 750 mM NaCl, 50 mM Tris-HCl pH 7.0.

Colorimetric activity measurements

To determine whether the added FX_a had been properly removed, both the Granzyme B and the FX_a activity was measured before and after removal of the added FX_a using a colorimetric assay with the substrates S2222 (N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroaniline, Chromogenix, Italy, cat. no. S2222) and Ac-IEPD-*p*NA (N-acetyl-L-isoleucyl-L-glutamyl-L-prolyl-L-aspartyl-*p*-nitroaniline, Calbiochem, La Jolla, USA, cat. no. 368067), where the absorbance was measured at 405 nm for approximately 3 minutes, and the ΔOD_{405}/min was calculated.

For the measurement of FX_a activity the following was mixed:
500 µl buffer, 25 µl 3 mM S2222, and 5 µl enzyme.

For the measurement of Granzyme B activity the following was mixed: 500 µl buffer, 2 µl 100 mM Ac-IEPD-*p*NA, and 5 µl enzyme.

The buffer used was either 100 mM NaCl, 50 mM Tris-HCl pH 8.0 or 100 mM HEPES pH 7.4. An example using the 100 mM NaCl, 50 mM Tris-HCl pH 8.0 buffer is shown below in table 3, where the top fraction from the SP Sepharose eluate after FX_a removal was used:

Table 3:

Before FX_a removal (ΔOD_{405}/min)		After FX_a removal (ΔOD_{405}/min)	
GrB activity	FX_a activity	GrB activity	FX_a activity
0.1401	0.0139	0.2213	0.0001

The activity of an incubation of GrB-H6 with FX_a was followed for several days using the following colorimetric assay: 500 µl buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0), 4 µl 100 mM Ac-IEPD-*p*NA, and 5 µl GrB-H6. The mixture of 100 µl GrB-H6 (approximately 10 µg) with 1 µl FX_a (1 mg/ml) was kept at 4°C during the incubation. The following data in table 4 was obtained. See also Figure 1.

Table 4:

Time	Time (hours)	ΔOD_{405}/min
0 hours	0	0.0073
2 hours	2	0.0200
5 hours	5	0.0250
19 hours	19	0.0829
2 days	45	0.1325
5 days	120	0.1832

<u>The effect of different buffers on the colorimetric assay</u>

The activity of the activated and purified GrB-H6 was measured in different buffers using the Ac-IEPD-*p*NA substrate: 500 µl buffer, 2 µl 100 mM Ac-

IEPD-*p*NA, and 5 µl GrB-H6. The ΔOD_{405}/min was calculated from the first 0.75 min unless otherwise noted. (TN = 100 mM NaCl, 50 mM Tris-HCl). The results are listed in table 5 and 6, where the measurements in table 6 were made with the same GrB-H6 batch as table 5, but with another 100 mM Ac-IEPD-*p*NA batch:

Table 5:

Buffer	Approximate amount of GrB-H6 added (µg)	Activity (ΔOD_{405}/min)	
TN pH 8.1	1	0.2213	(1 min)
TN pH 7.0	1	0.2794	
TN pH 7.4	1	0.2930	
		0.2624	
	0.5	0.0835	
		0.1082	
	0.2	0.0245	(3 min)
TN pH 7.4 + 0.1% TWEEN20	0.5	0.0887	
	0.2	0.0303	(3 min)
TN pH 7.4 + 5 mM Ca^{2+}	0.5	0.1401	
TN pH 7.4 + 5 mM Mg^{2+}	0.5	0.1491	
100 mM HEPES pH 7.5	0.5	0.2350	
100 mM HEPES pH 7.5 + 5 mM Ca^{2+}	0.5	0.2425	

Table 6:

Buffer	Approximate amount of GrB-H6 added (µg)	Activity (ΔOD_{405}/min)
100 mM HEPES pH 7.2	0.5	0.1970
100 mM HEPES pH 7.4	0.5	0.2273
		0.2328
100 mM HEPES pH 7.4 + 50 mM KCl	0.5	0.2167
100 mM HEPES pH 7.4 + 50 mM NaCl	0.5	0.1993
		0.1938
100 mM NaCl, 50 mM Tris-HCl pH 7.4]	0.5	0.1682
50 mM NaCl, 25 mM Tris-HCl pH 7.4	0.5	0.1948
100 mM KCl, 50 mM Tris-HCl pH 7.4	0.5	0.1662

It was found that 100 mM HEPES pH 7.4/7.5 was the best buffer for GrB-H6 activity of the buffers evaluated.

Example 4

Design and construction of expression vectors for fusions proteins containing an IEPD sequence cleavable by GrB-H6

In order to prepare suitable fusion proteins as substrates for Granzyme B, the FX_a recognition sequence in the FX_a cleavable fusion proteins H6-FX-TripBUB, H6-FX-RAP and H6-FX-TN123 (encoded by pT7H6-FX-TripBUB, pT7H6-FX-RAP and pT7H6-FX-TN123, respectively) was changed from either IEGR or IQGR to IEPD, giving the constructs H6-IEPD-TripUB (SEQ ID NO. 27), H6-IEPD-RAP (SEQ ID NO. 28), and H6-IEPD-TN123 (SEQ ID NO. 29). In addition, the IEPD↓SP cleavage site in the H6-TripUB construct was changed to four other cleavage sites to give the following variants: H6-TripUB IQAD↓SP (SEQ ID NO. 30), H6-TripUB IQAD↓SG (SEQ ID NO. 31), H6-TripUB VGPD↓SP (SEQ ID NO. 32) and H6-TripUB VGPD↓FG (SEQ ID NO. 33) where ↓ indicates the cleavage site. In two of these four constructs the P_1'

and P_2'-sites of the fusion protein were also changed, namely in H6-TripUB IQAD↓SG and H6-TripUB VGPD↓FG.

Construction of fusion protein expression vectors

The expression vector pT7H6-IEPD-TripUB was constructed by using the QuikChange™ Site-Directed Mutagenesis Kit (STRATAGENE, Catalog #200518) according to the manufacturers' protocol with the vector pT7H6-FX-TripBUB (WO9856906) as template and the oligonucleotide primers: TripUB GrB fw (SEQ ID NO: 12) and TripUB GrB rev (SEQ ID NO: 13).

The expression vector pT7H6-IEPD-RAP was constructed by site-directed mutagenesis as described above with the vector pT7H6-FX-RAP (Nykjær et al., 1992) as template and the oligonucleotide primers: RAP GrB fw (SEQ ID NO: 14) and RAP GrB rev (SEQ ID NO: 15).

The expression vector pT7H6-IEPD-TN123 was constructed by site-directed mutagenesis as described above with the vector pT7H6-FX-TN123 (Holtet et al., 1997) as template and the oligonucleotide primers: TN GrB fw (SEQ ID NO: 16) and TN GrB rev (SEQ ID NO: 17).

The expression vector pT7H6-TripUB IQAD↓SP was constructed by using site-directed mutagenesis as described above with the vector pT7H6-FX-TripBUB (WO 9856906) as template and the oligonucleotide primers: PC7TripUB GR-AD fw (SEQ ID NO: 18) and PC7TripUB GR-AD rev (SEQ ID NO: 19).

The expression vector pT7H6-TripUB IQAD↓SG was constructed by using site-directed mutagenesis as described above with the vector pT7H6-TripUB IQAD↓SP as template and the oligonucleotide primers: PC7TripUB P-G fw (SEQ ID NO: 20) and PC7TripUB P-G rev (SEQ ID NO: 21).

The expression vector pT7H6-TripUB VGPD↓SP was constructed by using site-directed mutagenesis as described above with the vector pT7H6-IEPD-TripUB as template and the oligonucleotide primers: DNATrip IE-VG fw (SEQ ID NO: 22) and DNATrip IE-VG rev (SEQ ID NO: 23).

The expression vector pT7H6-TripUB VGPD↓FG was constructed by using site-directed mutagenesis as described above with the vector pT7H6-TripUB VGPD↓SP as template and the oligonucleotide primers: DNATrip SP-FG fw (SEQ ID NO: 24) and DNATrip SP-FG rev (SEQ ID NO: 25).

Table 7: Oligonucleotide primers

Primer	Nucleotide sequence	SEQ ID NO.
TripUB GrB fw	5'-GTGGATCCATCGAGCCTGACTCTCCTGGTACCGAGCC-3'	12
TripUB GrB rev	5'-GGTACCAGGAGAGTCAGGCTCGATGGATCCACTACCAC-3'	13
RAP GrB fw	5'-CGGATCCATCGAGCCTGACTACTCGCGGGAGAAG-3'	14
RAP GrB rev	5'-CCCGCGAGTAGTCAGGCTCGATGGATCCGTGATG-3'	15
TN GrB fw	5'-GGATCCATCGAGCCTGACGGCGAGCCACCAACC-3'	16
TN GrB rev	5'-GGCTCGCCGTCAGGCTCGATGGATCCGTGATGG-3'	17
PC7TripUB GR-AD fw	5'-GGATCCATCCAGGCAGACTCTCCTGGTACCGAG-3'	18
PC7TripUB GR-AD rev	5'-GTACCAGGAGAGTCTGCCTGGATGGATCCACTAC-3'	19
PC7TripUB P-G fw	5'-GGATCCATCCAGGCAGACTCTGGTGGTACCGAGCCAC-3'	20
PC7TripUB P-G rev	5'-CTCGGTACCACCAGAGTCTGCCTGGATGGATCCACTAC-3'	21
DNATrip IE-VG fw	5'-GTAGTGGATCAGTCGGGCCTGACTCTCCTGGTAC-3'	22
DNATrip IE-VG rev	5'-GAGAGTCAGGCCCGACTGATCCACTACCACTACC-3'	23
DNATrip SP-FG fw	5'-GGCCTGACTTTGGTGGTACCGAGCCACCAAC-3'	24
DNATrip SP-FG rev	5'-GGCTCGGTACCACCAAAGTCAGGCCCGACTG-3'	25

Example 5

Expression, purification and refolding of fusion proteins containing the IEPD sequence cleavable by GrB-H6

Expression of the fusion proteins H6-IEPD-TripUB, H6-IEPD-RAP, H6-IEPD-TN123 and the H6-TripUB variants

To prepare the above chimeric fusion proteins H6-IEPD-TripUB, H6-IEPD-RAP, H6-IEPD-TN123 and the H6-TripUB variants, the vectors pT7H6-IEPD-TripUB, pT7H6-IEPD-RAP, pT7H6-IEPD-TN123, pT7H6-TripUB IQAD↓SP, pT7H6-TripUB IQAD↓SG, pT7H6-TripUB VGPD↓SP and pT7H6-TripUB VGPD↓FG (the last four termed H6-TripUB variants) were grown in a medium scale (3 litre; 2xTY medium, 5 mM $MgSO_4$ and 0.1 mg/ml ampicillin) in *E. coli* BL21 cells, as described by Studier FW et al. (1990). Exponentially growing cultures at 37°C were at OD_{600} = 0.8 infected with bacteriophage λCE6 at a multiplicity of approximately 5. Cultures were grown at 37°C for another four hours and the cells harvested by centrifugation. Cells were re-suspended in 100 ml of 750 mM NaCl, 100 mM Tris-HCl pH 8, and 1 mM EDTA pH 8. Phenol (150 ml adjusted to pH 8 with Trisma base) was added to each, and the mixtures were sonicated to extract total protein. After clarification by centrifugation (25 minutes at 10.000 g) crude protein fractions were precipitated from the phenol phases by addition of 2.5 volumes of 96 % ethanol and centrifugation. Protein pellets were dissolved in 75 ml 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 100 mM dithiothreitol (DTT).

Purification of H6-IEPD-TripUB, H6-IEPD-RAP and H6-TripUB variants

Following gel-filtration on Sephadex™ G-25 Fine (Amersham Biosciences) into 8 M Urea, 500 mM NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol, the crude protein preparations of the H6-IEPD-TripUB and H6-IEPD-RAP fusion proteins were applied by batch adsorption onto Ni^{2+} activated NTA-agarose (Ni^{2+}-NTA-agarose, Quiagen) columns (usually 50-75 ml column volume) for purification (Hochuli E et al., 1988). The column was washed with the following:

1. 2 x column volume 8 M urea, 500 mM NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol
2. 1 x column volume 8 M urea, 500 mM NaCl, 50 mM sodium-phosphate pH 6.3, and 10 mM 2-mercaptoethanol
3. 1 x column volume 6 M guanidinium chloride, and 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol
4. 2 x column volume 500 mM NaCl, and 50 mM Tris-HCl pH 8

The purified fusion proteins were then eluted with 500 mM NaCl, 50 mM Tris-HCl pH 8, and 10 mM EDTA.

Purification and refolding of H6-IEPD-TN123 fusion proteins

Following gel-filtration on Sephadex™ G-25 Fine (Amersham Biosciences) into 8 M Urea, 500 mM NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol, the crude protein preparations of the H6-IEPD-TN123 fusion proteins were applied by batch adsorption to Ni^{2+} activated NTA-agarose (Ni^{2+}-NTA-agarose, Quiagen) columns (usually 50-75 ml column volume) for purification and in vitro refolding. The column was washed with the following:

1. 2 x column volume 8 M urea, 500 mM NaCl, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol
2. 1 x column volume 8 M urea, 500 mM NaCl, 50 mM sodium-phosphate pH 6.3, and 10 mM 2-mercaptoethanol
3. 1 x column volume 6 M guanidinium chloride, 50 mM Tris-HCl pH 8, and 10 mM 2-mercaptoethanol

Each fusion protein was then subjected to the iterative refolding procedure as described for plasminogen kringle 4 by Thøgersen et al. (International Patent Application WO 9418227). After completion of the refolding procedure each refolded fusion protein was then eluted from the Ni^{2+}-NTA-agarose in 500 mM NaCl, 50 mM Tris-HCl pH 8, 10 mM EDTA.

Fractions of each refolded fusion protein was gel filtrated into 50 mM NaCl, 25 mM sodium acetate pH 5.0, and 1 mM $CaCl_2$, and was further purified by ion exchange chromatography on SP Sepharose™ Fast Flow (Amersham

Biosciences, 1.6 (i.d.) by 20 centimetre column) using a salt gradient from 50 mM NaCl, 25 mM sodium acetate pH 5.0 and 1 mM $CaCl_2$ to 1 M NaCl, 25 mM sodium acetate pH 5.0, 1 mM $CaCl_2$.

The final purification of each correctly folded fusion protein product was then accomplished by gel-filtration into 25 mM NaCl, 10 mM Tris-HCl pH 8, and 1 mM $CaCl_2$ followed by ion exchange chromatography on Q Sepharose™ Fast Flow (Amersham Biosciences, 1.6 (i.d.) by 20 centimetre column) using a salt gradient from 25 mM NaCl, 10 mM Tris-HCl pH 8, and 1 mM $CaCl_2$ to 500 mM NaCl, 10 mM Tris-HCl pH 8, and 1 mM $CaCl_2$.

Example 6

Cleavage of prepared fusion proteins by GrB-H6

Cleavage of H6-IEPD-TripUB by GrB-H6

The fusion protein H6-IEPD-TripUB (Example 5) eluted from the Ni^{2+}-NTA-agarose column was gel filtrated into 100 mM HEPES pH 7.5 and 200 µl samples of the top-fraction was incubated at room temperature with either 0, 1 or 10 µl of activated GrB-H6 (approximately 0, 0.2 and 2 µg GrB-H6).

Samples for SDS PAGE were taken after 12, 19, and 24 hours of incubation, and gels are shown in Figures 2 and 3.

Only the correctly cleaved product appear in lanes C-D in figure 2 and lanes C-G in figure 3, and the longer the incubation time, the more cleavage product appears in the lanes, both for the addition of 1 and 10 µl GrB-H6. The simple band pattern observed is explained in figure 4. From this it is clear that GrB-H6 cleaved H6-IEPD-TripUB specifically at a single site. Cleavage at the correct site after the IEPD sequence is confirmed in lane E in figure 2, where the construct H6-FX-TripUB, containing the FX_a recognition site IQGR in place of the GrB recognition site IEPD, was cleaved by FX_a giving a product of the same size as the GrB-H6 cleaved H6-IEPD-TripUB.

The effect of temperature and addition of Ni^{2+} and NTA on the cleavage of H6-IEPD-TripUB by GrB-H6

With the H6-IEPD-TripUB fusion protein the following nine incubations were set up using 200 µl H6-IEPD-TripUB and 5 µl GrB-H6 (approximately 1 µg GrB-H6) for each incubation.

Table 8:

1	No addition	23°C
2	4.2 mM Ni^{2+}	
3	4.2 mM Ni^{2+} + 5 mM NTA	
4	No addition	37°C
5	4.2 mM Ni^{2+}	
6	4.2 mM Ni^{2+} + 5 mM NTA	
7	No addition	42°C
8	4.2 mM Ni^{2+}	
9	4.2 mM Ni^{2+} + 5 mM NTA	

Samples for SDS PAGE were taken after 2, 7 and 22 hours of incubation, see Figures 5, 6 and 7.

It was contemplated that the Ni^{2+} ions would bind the N-terminal hexa-His tail (H6) of the fusion protein and facilitate access to the cleavage site recognized by GrB-H6. In addition the Ni^{2+} ions would also bind the C-terminal hexa-His tail of the GrB-H6 construct. The addition of NTA was made to shield the Ni^{2+} ions in solution in a similar fashion as on the Ni^{2+}-NTA agarose beads, i.e. to simulate the conditions on the Ni^{2+}-NTA agarose column.

Figure 5 shows the incubations at 23°C, figure 6 at 37°C and figure 7 at 42°C. When no Ni^{2+} or NTA was added, the H6-IEPD-TripUB fusion protein was cleaved similar to what is seen in figures 2 and 3, though after 22 hours it.

seems that incubation at 37°C is the most optimal of the three temperatures tested.

With the addition of 4.2 mM Ni^{2+} some protein precipitated at the higher temperatures of 37°C and 42°C, but no precipitation was seen at 23°C. Because of this no further cleavage of the fusion protein is seen in the gel after 2 hours incubation at these two temperatures, where it seems that both some H6-IEPD-TripUB and GrB-H6 precipitated. At 23°C more fusion protein was cleaved after 22 hours than with no addition of Ni^{2+}.

The observed precipitation problem was eliminated by the addition of 5 mM NTA to the incubations. After 22 hours incubation at 23°C more fusion protein had been cleaved than with no Ni^{2+} or NTA addition, so the addition of Ni^{2+} and NTA seems to speed up the cleavage reaction. By further increasing the temperature to 37°C and 42°C an even greater increase in the rate of cleavage is seen. After 22 hours of incubation at 37°C almost all the fusion protein was cleaved to the correct product. A little less was cleaved at 42°C after 22 hours.

Comparing the rate of cleavage initially estimated in the experiments shown in figures 2 and 3, to the rate of cleavage observed here, it is clear that the addition of Ni^{2+} and NTA as well as the incubation at 37°C speeds up the specific cleavage of H6-IEPD-TripUB by GrB-H6 dramatically.

Cleavage of H6-IEPD-RAP by GrB-H6

The fusion protein H6-IEPD-RAP (Example 5) eluted from the Ni^{2+}-NTA-agarose column was gel filtrated into 100 mM HEPES pH 7.4 and 200 µl samples of the top-fraction was incubated at room temperature with either 0, 1 or 10 µl of activated GrB-H6 (approximately 0, 0.2 and 2 µg GrB-H6). Samples for SDS PAGE were taken after 5, 23 and 26 hours of incubation, see Figure 8.

After only 5 hours incubation with either 1 or 10 µl GrB-H6 as described above all the H6-IEPD-RAP was cleaved to give the final product. It is also clear that there is at least one internal cleavage site in RAP, but this internal site was cleaved much slower than the IEPD sequence, though. That GrB-H6 cleaved off the H6 correctly at the IEPD sequence can be seen by comparing the size of the product with purified samples of H6-FX-RAP cleaved partly (lane H) or completely (lane I) by FX_a to give the final RAP product. In these lanes the degradation products from any internal cleavage by FX_a had been removed by purification.

Comparison of H6-GrB-RAP cleavage by GrB-H6 and H6-FX-RAP cleavage by FXa

The cleavage of H6-GrB-RAP by GrB-H6 was compared with the cleavage of H6-FX-RAP by FX_a. Both H6-GrB-RAP and H6-FX-RAP were in 100 mM HEPES pH 7.4 and the following incubations were set up at room temperature:

1. 400 µl H6-FX-RAP + 1 µl FX_a (1 mg/ml)
2. 400 µl H6-GrB-RAP + 2 µl GrB-H6 (app. 0.2 µg)

Samples were taken for SDS PAGE after 0, ½, 1, 3, 5, 7 and 27 hours of incubation, see Figure 9.

It is clear that both fusion proteins were cleaved very rapidly by their respective protease, so that after only ½ hour almost all of the fusion protein had been cleaved to give the correct product for both incubations.

For the H6-FX-RAP + FX_a incubation, though, all of the fusion protein had been degraded to give a variety of smaller pieces after 27 hours, and there is no correctly cleaved product left.

In the H6-IEPD-RAP + GrB-H6 incubation degradation products show up as for the H6-FX-RAP incubation, but not as many as for the H6-FX-RAP

incubation. There seems to be only one GrB-sensitive site in RAP, while there are several FX_a sensitive sites. This slows down the degradation of H6-GrB-RAP by GrB-H6, whereby quite a lot of correctly cleaved product is still present after 27 hours of incubation.

In summary the correct cleavage of the RAP fusion protein by GrB-H6 is just as fast as by FX_a, but the degradation of the RAP fusion protein by GrB-H6 is much slower than the degradation by FX_a.

Cleavage of H6-IEPD-TN123 by GrB-H6

The fusion protein H6-IEPD-TN123 (Example 5) eluted from the Q Sepharose was after final purification gel filtrated into 100 mM HEPES pH 7.5, and 200 µl samples of the top-fraction was incubated at room temperature with either 0, 1 or 10 µl of activated GrB-H6 (approximately 0, 0.2 and 2 µg GrB-H6) both with and without 5 mM $CaCl_2$ present. Samples for SDS PAGE from the incubations without $CaCl_2$ were taken after 12, 19 and 24 hours as well as 5 days of incubation. See Figures 2, 3 and 10. Samples for SDS PAGE from the incubations both with and without $CaCl_2$ were taken after approximately 20 and 48 hours of incubation, see figure 11.

Without Ca^{2+}:

The samples showed a distinct band pattern when H6-IEPD-TN123 was cleaved by GrB-H6 with no Ca^{2+} present, as seen in figures 2, 3 and 10. The H6-IEPD-TN123 was cleaved correctly at the IEPD sequence, but also just as rapidly at an internal site of the sequence AQPD. The band pattern is explained in figure 12. That H6-IEPD-TN123 was cleaved at the correct IEPD↓ site can be seen from lane J in figure 2, where murine H6-FX-TN123 had been cleaved by FX_a giving a product of the same size as the product from GrB-H6 cleavage of H6-IEPD-TN123 with no internal cleavage, i.e. the lowest band of the four bands in the pattern.

When the samples were reduced as in lanes B-D and L-N in figure 10 a different band pattern appears. This pattern is also explained in Figure 12 and supports the notion of the specific internal cleavage site AQPD.

With Ca^{2+}:

Figure 11 shows incubations of H6-IEPD-TN123 with GrB-H6, where 5 mM $CaCl_2$ were added to some of the incubations. Here only two bands appear when Ca^{2+} is present (lanes E and G), while four bands appear when no Ca^{2+} is present, as described for Figures 2, 3, 10 and 12. This shows that by adding Ca^{2+} to the incubation the internal cleavage site AQPD in Tetranectin (TN123) can be made inaccessible to GrB-H6. This is because the AQPD sequence is located in a loop, where the Q and D residues participates in the binding of Ca^{2+}-ions in Tetranectin. Thereby only the correct cleavage at the specific IEPD site in the fusion protein occurs, and the internal cleavage site in TN123 is "turned off" by the addition of Ca^{2+}.

Cleavage of the H6-TripUB variants

Each of the fusion proteins eluted from the Ni^{2+}-NTA-agarose column were gel filtrated into 100 mM HEPES pH 7.4 and fractions of approximately the same concentration of the five different H6-TripUB variants were used. The five variants were H6-IEPD-TripUB, H6-TripUB IQAD↓SP, H6-TripUB IQAD↓SG, H6-TripUB VGPD↓SP and H6-TripUB VGPD↓FG. Of each fusion protein 200 µl was incubated at room temperature with 5 µl of activated GrB-H6 (approximately 1 µg GrB-H6). Samples for SDS PAGE were taken after 2, 6, 24 and 48 hours of incubation and gels are shown in Figures 13 and 14.

In Figure 13 are shown the samples of H6-IEPD-TripUB, H6-TripUB IQAD↓SP and H6-TripUB IQAD↓SG. After 48 hours incubation approximately 2/3 of the original amount of non-cleaved H6-IEPD-TripUB had been correctly cleaved. In comparison the cleavage of the sequence IQAD↓SP, though, was much slower than cleavage of the IEPD↓SP sequence in H6-IEPD-TripUB. No product is visible after 2 hours incubation and only a small amount had been cleaved after 48 hours incubation. From the H6-TripUB IQAD↓SG

samples it is evident that the cleavage was much faster than for both H6-IEPD-TripUB and H6-TripUB IQAD↓SP. Already after 2 hours incubation the majority of the fusion protein had been cleaved to give the correct product. The single mutation of Pro (P) to Gly (G) in the P'_2 site in the recognition sequence was enough for this dramatic change in the cleavage rate.

In figure 14 are shown the samples from the H6-TripUB VGPD↓SP and H6-TripUB VGPD↓FG incubations. The cleavage of the VGPD↓SP sequence was almost as fast as for H6-IEPD-TripUB in figure 13. A small amount of product had formed after 2 hours and approximately half the amount of fusion protein had been cleaved after 48 hours. A dramatic change in reaction rate occured when the P'_1 and P'_2 sites were changed from SP to FG in H6-TripUB VGPD↓SP. After only 2 hours incubation all the fusion protein had been correctly cleaved.

Comparing this to the results from the H6-IEPD-RAP incubations, in which the P'_1 site is a Tyr, it seems that the cleavage by GrB-H6 is much faster when the P'_1 site is a large aromatic residue and/or the P'_2 site is a Gly. In addition to these observations it is also important to note that even after 48 hours incubation with GrB-H6 no internal cleavage took place in any of the H6-TripUB variants, showing that GrB-H6 cleaves very specifically at the engineered recognition sites, even though the TripUB sequence contains 7 other Asp (D) residues.

Self-activating human Granzyme B

Recombinant self-activating human Granzyme B derivatives IEPD-GrB-H6 and IEAD-GrB-H6 were produced as described for GrB-H6 in Example 3 by using the expression vectors described in Example 2.

The IEAD-GrB-H6 and IEPD-GrB-H6 proteins eluted from the SP Sepharose columns were stored at 4°C for 2 days before the activity of the respective topfractions were determined by using a colorimetric assay. For this purpose the following was mixed: 500 µl buffer (100 mM HEPES pH 7.5), 2 µl 100 mM

Ac-IEPD-*p*NA, and 5 µl protein solution. The change in absorption was then determined at 405 nm during 3 min. The activity was further determined after additional incubation for 1 and 2 days at 4°C. The following data shown in Table 9 was obtained.

Table 9:

Protein		ΔOD_{405}/min
IEAD-GrB-H6	2 days	0.1372
IEPD-GrB-H6	2 days	0.1284
IEAD-GrB-H6	3 days	0.1607
IEPD-GrB-H6	3 days	0.1375
IEAD-GrB-H6	4 days	0.1983
IEPD-GrB-H6	4 days	0.1351

This means that the self-activating derivatives IEPD-GrB-H6 and IEAD-GrB-H6 were activated without addition of any previously activated GrB and the activation was not finished until after a least three or four days at 4°C.

References

Christensen JH et al. (1991), FEBS Letters, 281(1-2): 181-184.

Nykjær A et al. (1992), Journal of Biological Chemistry, 267(21): 14543-14548.

Holtet TL et al. (1997), Protein Science, 6: 1511-1515

Studier F.W. et al. (1990), Methods in Ezymology, 185: 60-89

Hochuli E et al. (1988), Biotechnology, 1321-1325

Harris et al (1998), Journal of Biological Chemistry 273(42): 27364-27373.

Casciola-Rosen et al. (1999), Journal of Experimental Medicine 190(6): 815–825.

Thøgersen et al., International Patent Application WO 9418227

Sun J et al (2001), Journal of Biological Chemistry 276(18): 15177-15184.

Claims

1. A method for the preparation of a polypeptide of interest in authentic form, said method comprising the steps of:

(i) providing a fusion protein comprising a polypeptide of interest and a fusion partner wherein the junction region between said polypeptide of interest and said fusion partner comprises a Granzyme B protease recognition site which comprises a Granzyme B protease cleavage site adjacent to the protein of interest, and

(ii) contacting said fusion protein with Granzyme B protease (EC 3.4.21.79) to cleave it at said cleavage site to yield said polypeptide of interest in authentic form.

2. A method according to claim 1, wherein the Granzyme B protease recognition site has an amino acid sequence of the general formula:

P4 P3 P2 P1↓

wherein

P4 is amino acid I or V
P3 is amino acid E, Q or M
P2 is X, where X denotes any amino acid,
P1 is amino acid D, and
↓ is the cleavage site for said Granzyme B protease.

3. A method according to claim 1, wherein the Granzyme B protease recognition site has an amino acid sequence selected from the group consisting of ICPD↓, IEAD↓, IEPD↓, IETD↓, IQAD↓, ISAD↓, ISSD↓, ITPD↓, VAPD↓, VATD↓, VCTD↓, VDPD↓, VDSD↓, VEKD↓, VEQD↓, VGPD↓, VRPD↓, VTPD↓, LEED↓, LEID↓, LGND↓, LGPD↓, AQPD↓, DEVD↓, and wherein ↓ is the cleavage site for said Granzyme B protease.

4. A method according to claim 2, wherein the general formula furthermore comprises the amino acids P1' and P2' resulting in the general formula P4 P3 P2 P1↓P1'P2', wherein P1' is X where X denotes any amino acid, P2' is G, and wherein P1' and P2' is a part of the protein of interest.

5. A method according to claim 2, wherein the general formula furthermore comprises the amino acids P1', P2', P3' and P4' resulting in the general formula P4 P3 P2 P1↓P1'P2'P3'P4', wherein P4' is D or E, and wherein P1', P2', P3' and P4' is a part of the protein of interest.

6. A method according to claim 1, wherein the protein of interest is selected from the group consisting of an enzyme and a polypeptide hormone.

7. A method according to claim 6, wherein the polypeptide hormone is selected from the group consisting of somatotrophin, glucagon, insulin and inteferon.

8. A method according to claim 6, wherein the enzyme is Granzyme B.

9. A method according to claim 1, wherein the fusion partner is an affinity-tag.

10. A method according to claim 9, wherein the affinity-tag is selected from the group consisting of a polyhistidine-tag, a polyarginine-tag, a FLAG-tag, a Strep-tag, a c-myc-tag, a S-tag, a calmodulin-binding peptide, a cellulose-binding peptide, a chitin-binding domain, a glutathione S-transferase-tag, and a maltose binding protein.

11. A method according to claim 1, wherein the Granzyme B protease is selected from the group consisting of human Granzyme B protease, mouse Granzyme B protease and rat Granzyme B protease.

12. A method according to claim 1, wherein the Granzyme B protease is in an immobilised form.

13. A method according to claim 12, wherein the Granzyme B protease is immobilised via the C-terminus.

14. A method according to claim 12, wherein the Granzyme B protease is immobilised via a lysine amino acid residue.

15. A method according to claim 10, wherein the tag is a polyhistidine-tag, and wherein the fusion protein is contacted with Granzyme B protease in the presence of Ni^{2+} ions and Nitrilotriacetic Acid (NTA).

16. A method according to claim 15, wherein the concentration of Ni^{2+} is in the range of 1-20 mM, and the concentration of NTA is in the range of 1-20 mM.

17. A fusion protein comprising a polypeptide of interest and a fusion partner, wherein the junction region between said polypeptide of interest and said fusion partner comprises a Granzyme B protease recognition site, which comprises a Granzyme B protease cleavage site adjacent to the protein of interest.

18. A fusion protein according to claim 17, wherein the Granzyme B protease recognition site has an amino acid sequence of the general formula:

P4 P3 P2 P1↓

wherein

P4 is amino acid I or V

P3 is amino acid E, Q or M

P2 is X, where X denotes any amino acid,

P1 is amino acid D, and

↓ is the cleavage site for said Granzyme B protease.

19. A fusion protein according to claim 17, wherein the Granzyme B protease recognition site has an amino acid sequence selected from the group consisting of ICPD↓, IEAD↓, IEPD↓, IETD↓, IQAD↓, ISAD↓, ISSD↓, ITPD↓,

VAPD↓, VATD↓, VCTD↓, VDPD↓, VDSD↓, VEKD↓, VEQD↓, VGPD↓, VRPD↓, VTPD↓, LEED↓, LEID↓, LGND↓, LGPD↓, AQPD↓, DEVD↓, and wherein ↓ is the cleavage site for said Granzyme B protease.

20. A fusion protein according to claim 18, wherein the general formula furthermore comprises the amino acids P1' and P2' resulting in the general formula P4 P3 P2 P1↓P1'P2', wherein P1' is X where X denotes any amino acid, P2' is G, and wherein P1' and P2' is a part of the polypeptide of interest.

21. A fusion protein according to claim 18, wherein the general formula furthermore comprises the amino acids P1', P2', P3' and P4' resulting in the general formula P4 P3 P2 P1↓P1'P2'P3'P4', wherein P4' is D or E, and wherein P1', P2', P3' and P4' is a part of the protein of interest.

22. A fusion protein according to claim 17, wherein the polypeptide of interest is selected from the group consisting of an enzyme and a polypeptide hormone.

23. A fusion protein according to claim 22, wherein the polypeptide hormone is selected from the group consisting of somatotrophin, glucagon, insulin and inteferon.

24. A fusion protein according to claim 22, wherein the enzyme is Granzyme B.

25. A fusion protein according to claim 17, wherein the fusion partner is an affinity-tag.

26. A fusion protein according to claim 25, wherein the affinity-tag is selected from the group consisting of a polyhistidine-tag, a polyarginine-tag, a FLAG-tag, a Strep-tag, a c-myc-tag, a S-tag, a calmodulin-binding peptide, a cellulose-binding peptide, a chitin-binding domain, a glutathione S-transferase-tag, and a maltose binding protein.

27. An isolated nucleic acid sequence encoding the fusion protein according to any of claims 18-26.

28. A recombinant vector comprising the isolated nucleic acid sequence according to claim 27.

29. A host cell transformed with a vector according to claim 28.

30. A method for the production of a fusion protein according to claim 17 comprising the steps of:
(i) providing a recombinant vector comprising the isolated nucleic acid sequence according to claim 27 operatively linked to a promotor,
(ii) transforming a host cell with said recombinant vector,
(iii) culturing said host cell under conditions to express said fusion protein, and
(iv) optionally isolating said fusion protein.

Abstract

There is provided a method for the preparation of a polypeptide of interest in authentic form by enzymatic cleavage of fusion proteins using Granzyme B protease (EC 3.4.21.79). There is also provided fusion proteins comprising a polypeptide of interest and a fusion partner, wherein the junction region between the polypeptide of interest and the fusion partner comprises a Granzyme B protease cleavage site adjacent to the protein of interest.

Figure 1

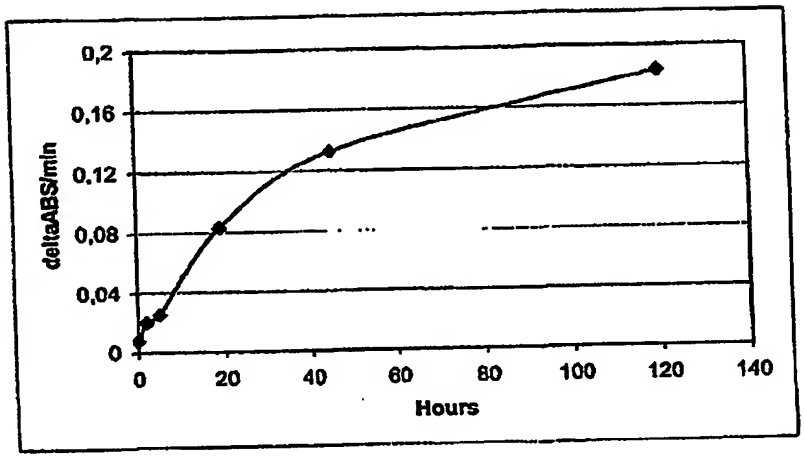


Figure 2

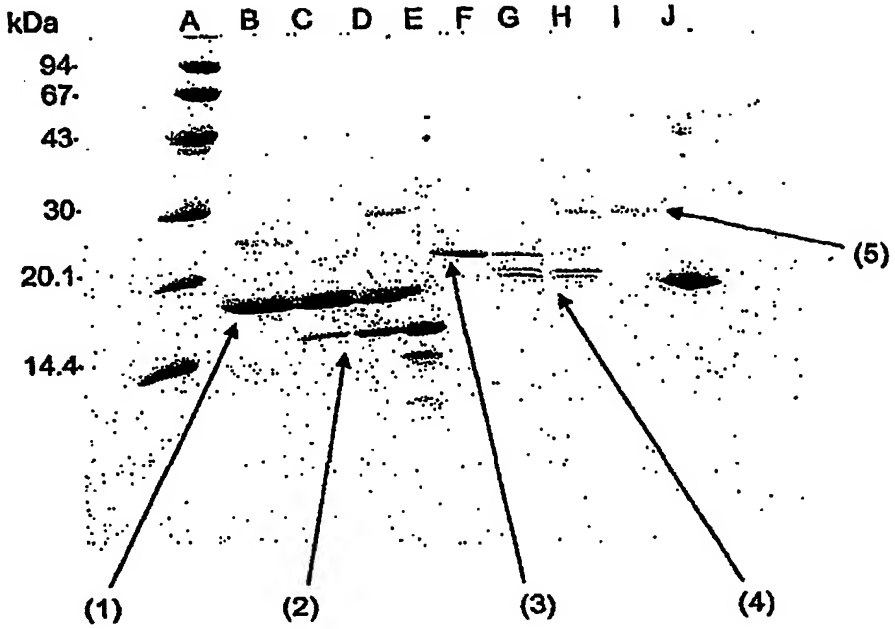


Figure 3

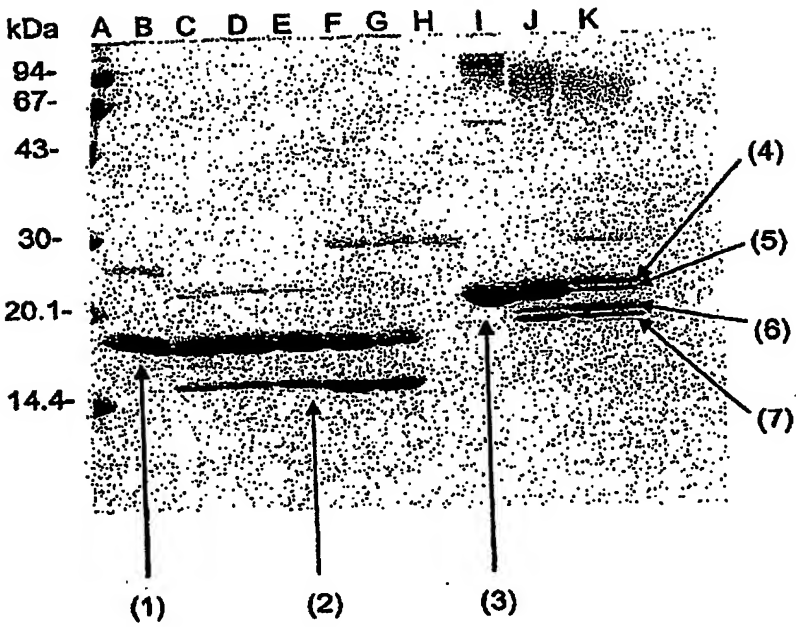


Figure 4

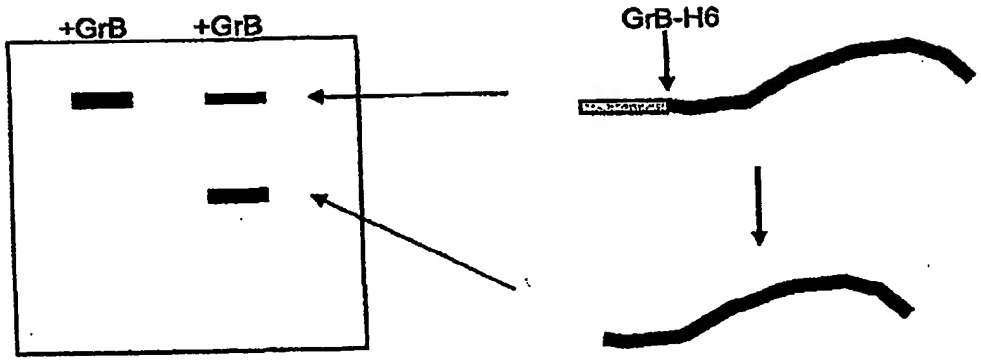


Figure 5

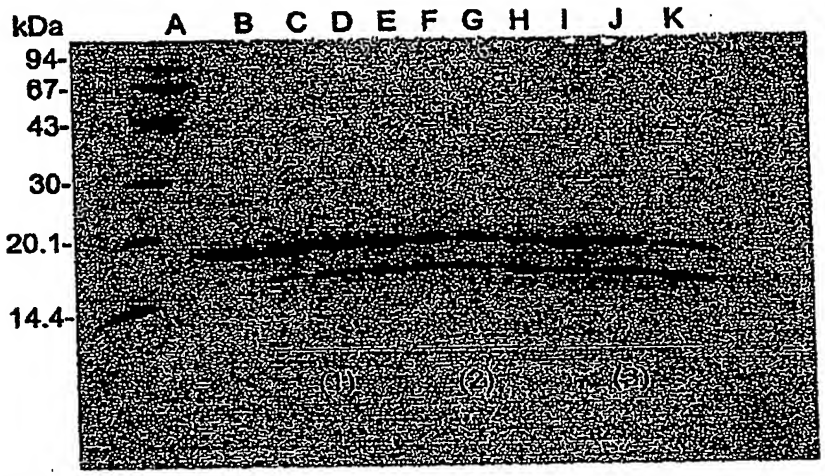


Figure 6

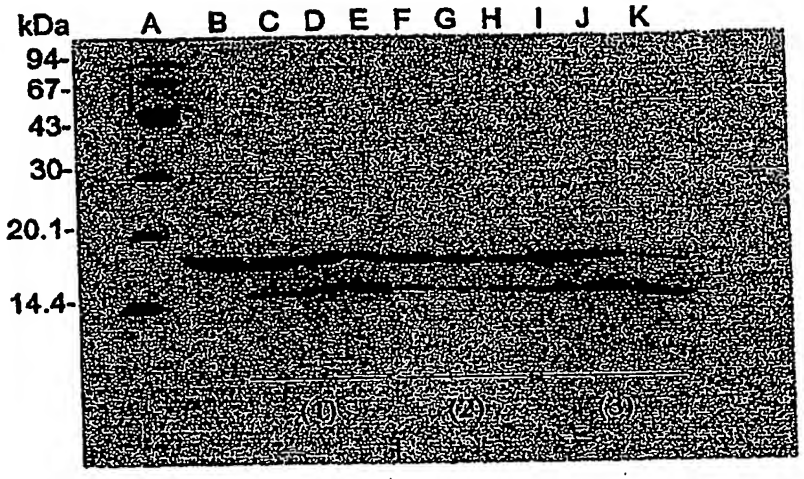


Figure 7

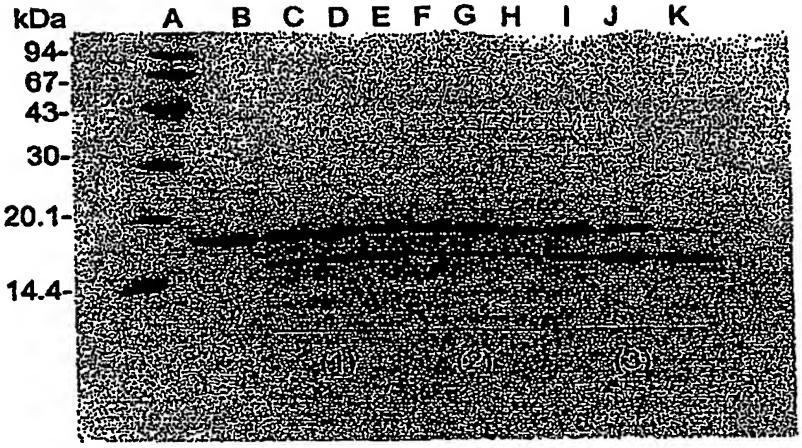


Figure 8

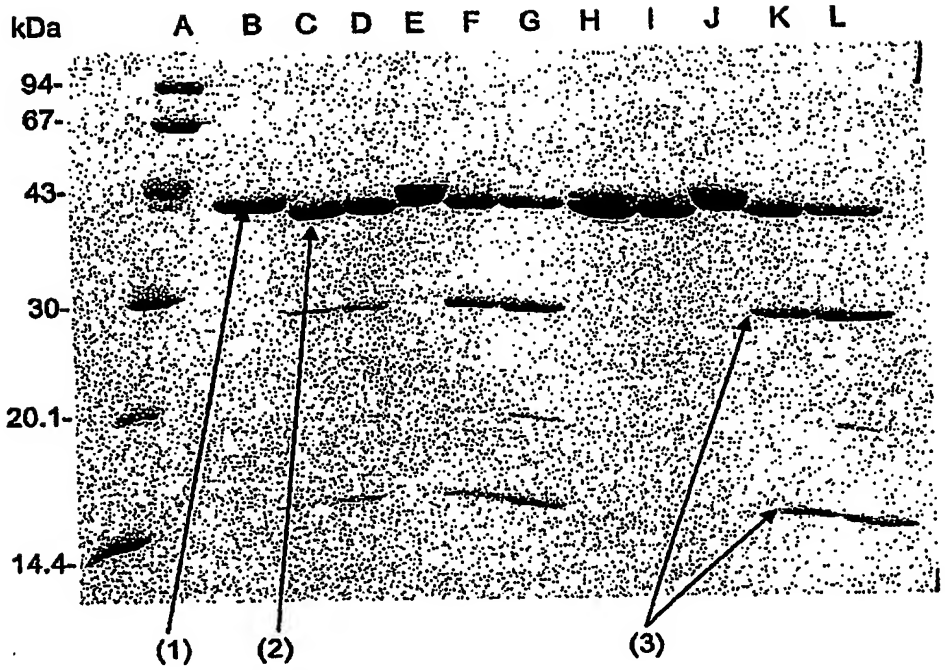


Figure 9

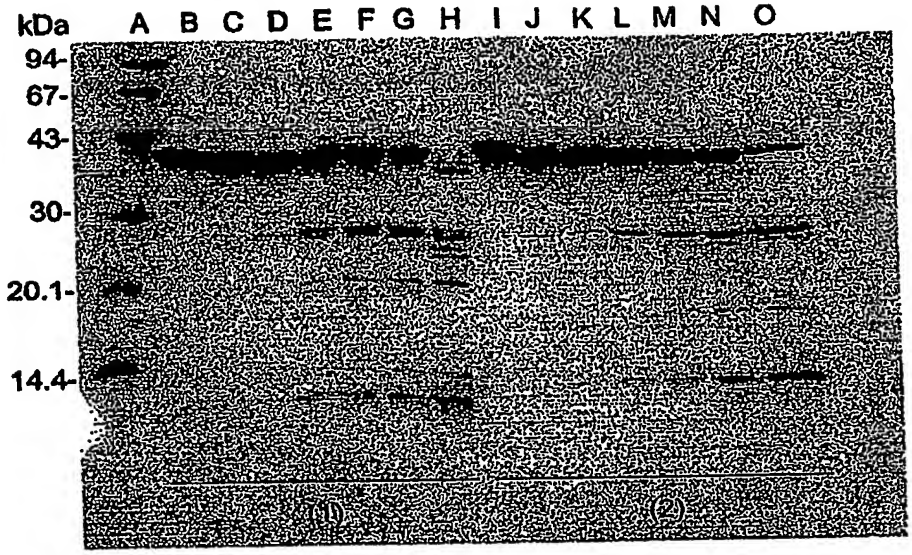


Figure 10

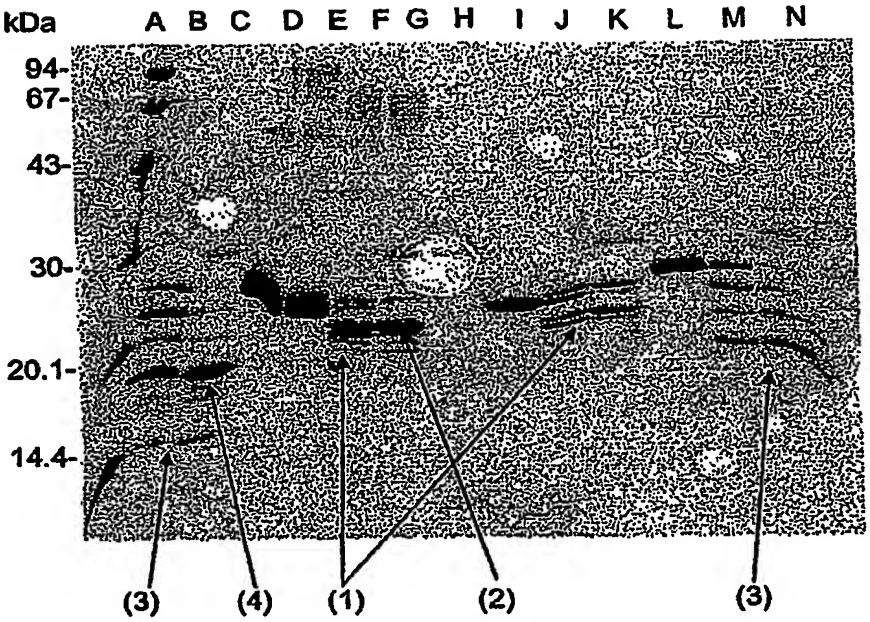


Figure 11

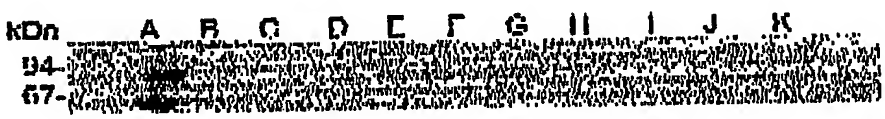


Figure 12

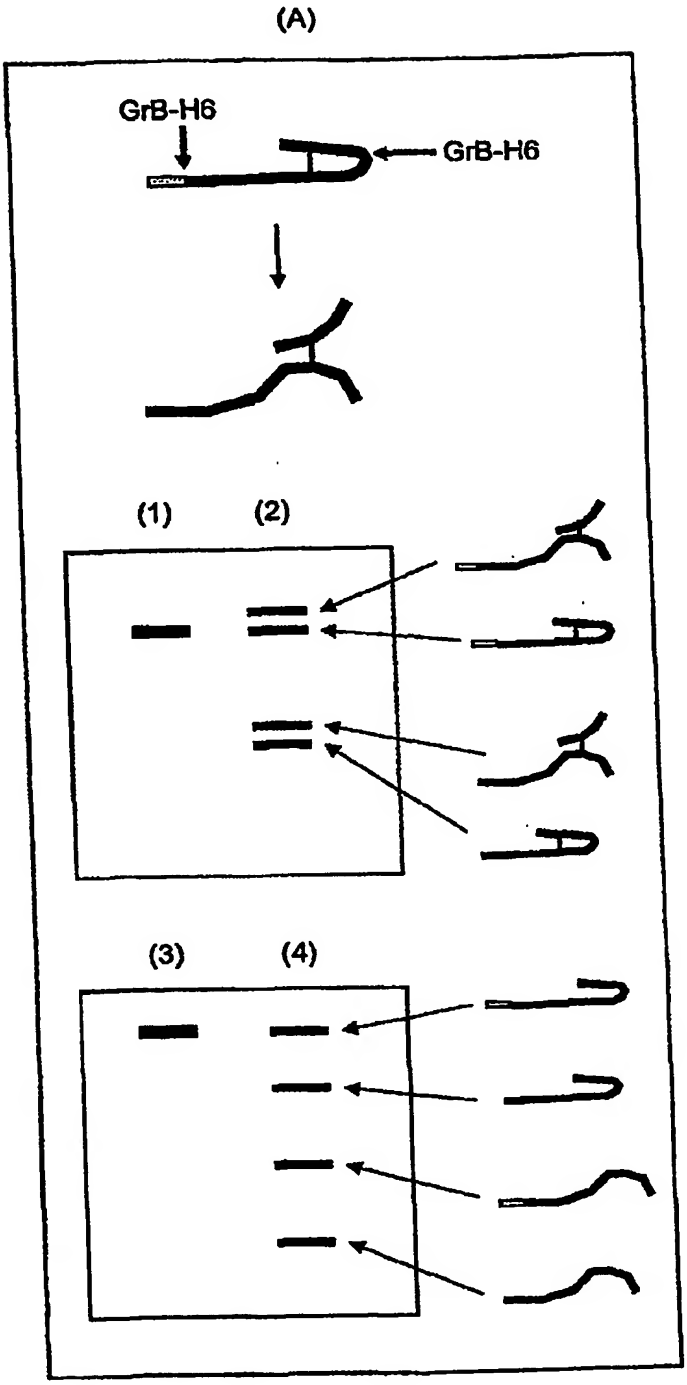


Figure 12 continued

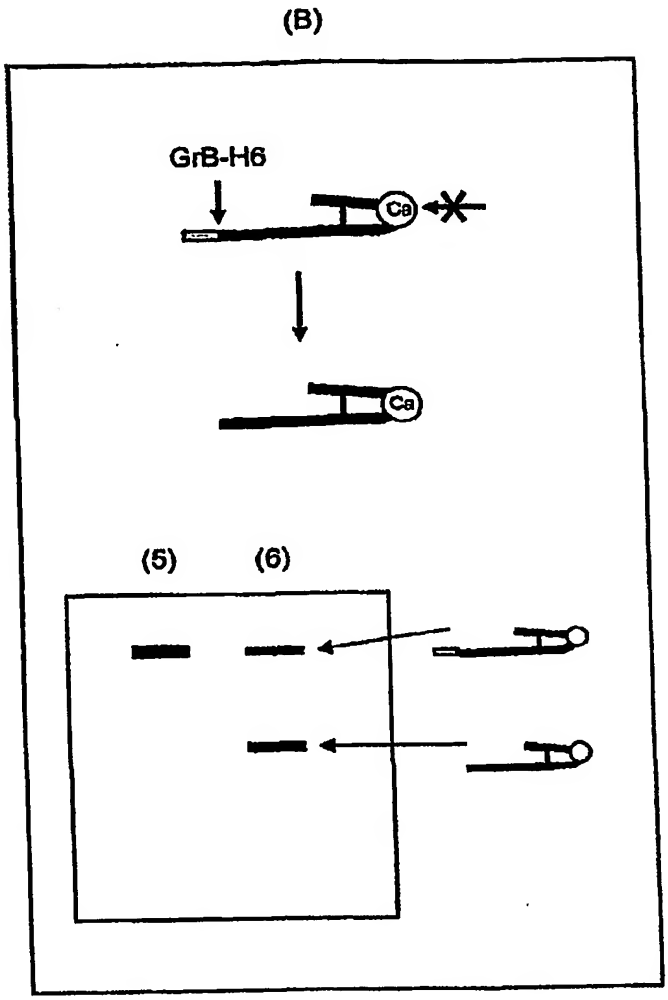


Figure 13

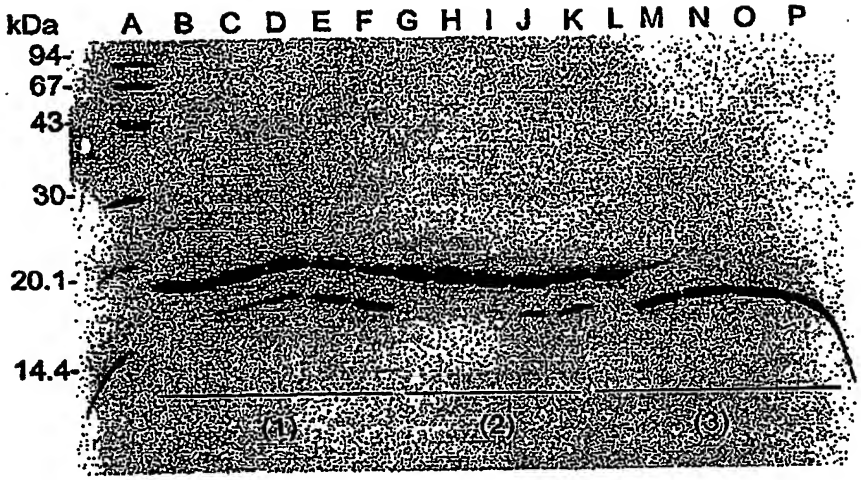
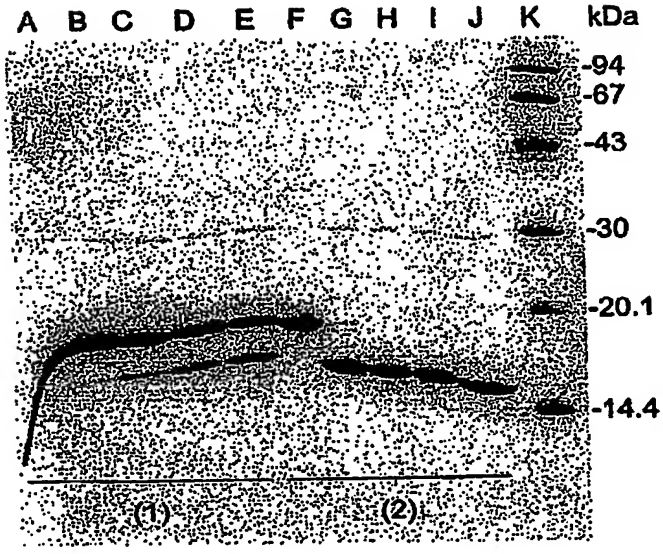


Figure 14



SEQUENCE LISTING

<110> Borean Pharma A/S

<120> Cleavage of fusion proteins using Granzyme B protease

<130> 3106

<160> 33

<170> PatentIn version 3.1

<210> 1
<211> 46
<212> DNA
<213> Artificial

<220>
<223> H6 C-term fw

<400> 1
catggacgga agcttgaatt cacatcacca tcaccatcac taacgc 46

<210> 2
<211> 46
<212> DNA
<213> Artificial

<220>
<223> H6 C-term rev

<400> 2
aattgcgtta gtgatggtga tggtgatgtg aattcaagct tccgct 46

<210> 3
<211> 40
<212> DNA
<213> Artificial

<220>
<223> GrBfw primer

<400> 3
catgggatcc atcgagggta ggatcatcgg gggacatgag 40

<210> 4
<211> 38
<212> DNA
<213> Artificial

<220>
<223> GrBrev EcoRI primer

<400> 4
gcgtgaattc aggtaccgtt tcatggtttt ctttatcc 38

<210> 5
<211> 715
<212> DNA
<213> Artificial

<220>
<223> GrB EcoRI fragment

<400> 5

catgggatcc atcgagggta ggatcatcgg gggacatgag gccaagcccc actcccgccc 60
ctacatggct tatcttatga tctgggatca gaagtctctg aagaggtgcg gtggcttcct 120
gatacaagac gacttcgtgc tgacagctgc tcactgttgg ggaagctcca taaatgtcac 180
cttgggggcc cacaatatca aagaacagga gccgacccag cagtttatcc ctgtgaaaag 240
acccatcccc catccagcct ataatcctaa gaacttctcc aacgacatca tgctactgca 300
gctggagaga aaggccaagc ggaccagagc tgtgcagccc ctcaggctac ctagcaacaa 360
ggcccaggtg aagccagggc agacatgcag tgtggccggc tgggggcaga cggccccccct 420
gggaaaacac tcacacacac tacaagaggt gaagatgaca gtgcaggaag atcgaaagtg 480
cgaatctgac ttacgccatt attacgacag taccattgag ttgtgcgtgg gggacccaga 540
gattaaaaag acttccttta agggggactc tggaggccct cttgtgtgta acaaggtggc 600
ccagggcatt gtctcctatg gacgaaacaa tggcatgcct ccacgagcct gcaccaaagt 660
ctcaagcttt gtacactgga taaagaaaac catgaaacgg tacctgaatt cacgc 715

<210> 6
<211> 30
<212> DNA
<213> Artificial

<220>
<223> GrBrev HindIII primer

<400> 6
ggcgaagctt agtagcgttt catggttttc 30

<210> 7
<211> 714
<212> DNA
<213> Artificial

<220>
<223> GrB HindIII fragment

<400> 7
catgggatcc atcgagggta ggatcatcgg gggacatgag gccaagcccc actcccgccc 60
ctacatggct tatcttatga tctgggatca gaagtctctg aagaggtgcg gtggcttcct 120
gatacaagac gacttcgtgc tgacagctgc tcactgttgg ggaagctcca taaatgtcac 180
cttgggggcc cacaatatca aagaacagga gccgacccag cagtttatcc ctgtgaaaag 240
acccatcccc catccagcct ataatcctaa gaacttctcc aacgacatca tgctactgca 300
gctggagaga aaggccaagc ggaccagagc tgtgcagccc ctcaggctac ctagcaacaa 360
ggcccaggtg aagccagggc agacatgcag tgtggccggc tgggggcaga cggccccccct 420
gggaaaacac tcacacacac tacaagaggt gaagatgaca gtgcaggaag atcgaaagtg 480
cgaatctgac ttacgccatt attacgacag taccattgag ttgtgcgtgg gggacccaga 540
gattaaaaag acttccttta agggggactc tggaggccct cttgtgtgta acaaggtggc 600
ccagggcatt gtctcctatg gacgaaacaa tggcatgcct ccacgagcct gcaccaaagt 660
ctcaagcttt gtacactgga taaagaaaac catgaaacgc tactaagctt cgcc 714

<210> 8
<211> 33
<212> DNA
<213> Artificial

<220>
<223> GrB GR-PD fw

<400> 8
tccatcgagc cggatatcat cgggggacat gag 33

<210> 9
<211> 34
<212> DNA
<213> Artificial

<220>
<223> GrB GR-PD rev

<400> 9
ccccgatgat atccggctcg atggatccca tatg 34

<210> 10
<211> 33
<212> DNA
<213> Artificial

<220>
<223> GrB GR-AD fw

<400> 10
tccatcgagg ctgatatcat cgggggacat gag 33

<210> 11
<211> 34
<212> DNA
<213> Artificial

<220>
<223> GrB GR-AD rev

<400> 11
ccccgatgat atcagcctcg atggatccca tatg 34

<210> 12
<211> 37
<212> DNA
<213> Artificial

<220>
<223> TripUB GrB fw primer

<400> 12
gtggatccat cgagcctgac tctcctggta ccgagcc 37

<210> 13
<211> 38
<212> DNA
<213> Artificial

<220>
<223> TripUB GrB rev primer

3

<400> 13
ggtaccagga gagtcaggct cgatggatcc actaccac 38

<210> 14
<211> 34
<212> DNA
<213> Artificial

<220>
<223> RAP GrB fw primer

<400> 14
cggatccatc gagcctgact actcgcggga gaag 34

<210> 15
<211> 34
<212> DNA
<213> Artificial

<220>
<223> RAP GrB rev primer

<400> 15
cccgcgagta gtcaggctcg atggatccgt gatg 34

<210> 16
<211> 33
<212> DNA
<213> Artificial

<220>
<223> TN GrB fw primer

<400> 16
ggatccatcg agcctgacgg cgagccacca acc 33

<210> 17
<211> 33
<212> DNA
<213> Artificial

<220>
<223> TN GrB rev primer

<400> 17
ggctcgccgt caggctcgat ggatccgtga tgg 33

<210> 18
<211> 33
<212> DNA
<213> Artificial

<220>
<223> PC7TripUB GR-AD fw

<400> 18
ggatccatcc aggcagactc tcctggtacc gag 33

<210> 19
<211> 34
<212> DNA
<213> Artificial

<220>
<223> PC7TripUB GR-AD rev

<400> 19
gtaccaggag agtctgcctg gatggatcca ctac 34

<210> 20
<211> 37
<212> DNA
<213> Artificial

<220>
<223> PC7TripUB P-G fw

<400> 20
ggatccatcc aggcagactc tggtggtacc gagccac 37

<210> 21
<211> 38
<212> DNA
<213> Artificial

<220>
<223> PC7TripUB P-G rev

<400> 21
ctcggtacca ccagagtctg cctggatgga tccactac 38

<210> 22
<211> 34
<212> DNA
<213> Artificial

<220>
<223> DNATrip IE-VG fw

<400> 22
gtagtggatc agtcgggcct gactctcctg gtac 34

<210> 23
<211> 34
<212> DNA
<213> Artificial

<220>
<223> DNATrip IE-VG rev

<400> 23
gagagtcagg cccgactgat ccactaccac tacc 34

<210> 24
<211> 31
<212> DNA
<213> Artificial

<220>
<223> DNATrip SP-FG fw

<400> 24
ggcctgactt tggtggtacc gagccaccaa c 31

<210> 25

<211> 31
<212> DNA
<213> Artificial

<220>
<223> DNATrip SP-FG rev

<400> 25
ggctcggtac caccaaagtc aggcccgact g 31

<210> 26
<211> 243
<212> PRT
<213> Artificial

<220>
<223> GrB-H6

<400> 26

Met Gly Ser Ile Glu Gly Arg Ile Ile Gly Gly His Glu Ala Lys Pro
1 5 10 15

His Ser Arg Pro Tyr Met Ala Tyr Leu Met Ile Trp Asp Gln Lys Ser
20 25 30

Leu Lys Arg Cys Gly Gly Phe Leu Ile Gln Asp Asp Phe Val Leu Thr
35 40 45

Ala Ala His Cys Trp Gly Ser Ser Ile Asn Val Thr Leu Gly Ala His
50 55 60

Asn Ile Lys Glu Gln Glu Pro Thr Gln Gln Phe Ile Pro Val Lys Arg
65 70 75 80

Pro Ile Pro His Pro Ala Tyr Asn Pro Lys Asn Phe Ser Asn Asp Ile
85 90 95

Met Leu Leu Gln Leu Glu Arg Lys Ala Lys Arg Thr Arg Ala Val Gln
100 105 110

Pro Leu Arg Leu Pro Ser Asn Lys Ala Gln Val Lys Pro Gly Gln Thr
115 120 125

Cys Ser Val Ala Gly Trp Gly Gln Thr Ala Pro Leu Gly Lys His Ser
130 135 140

His Thr Leu Gln Glu Val Lys Met Thr Val Gln Glu Asp Arg Lys Cys
145 150 155 160

Glu Ser Asp Leu Arg His Tyr Tyr Asp Ser Thr Ile Glu Leu Cys Val
165 170 175

Gly Asp Pro Glu Ile Lys Lys Thr Ser Phe Lys Gly Asp Ser Gly Gly
180 185 190

Pro Leu Val Cys Asn Lys Val Ala Gln Gly Ile Val Ser Tyr Gly Arg
195 200 205

Asn Asn Gly Met Pro Pro Arg Ala Cys Thr Lys Val Ser Ser Phe Val
210 215 220

His Trp Ile Lys Lys Thr Met Lys Arg Tyr Leu Asn Ser His His His
225 230 235 240

His His His

<210> 27
<211> 150
<212> PRT
<213> Artificial

<220>
<223> H6-IEPD-TripUB

<400> 27

Met Gly Ser His His His His His His Gly Ser Gly Ser Gly Ser Ile
1 5 10 15

Glu Pro Asp Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys
20 25 30

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
35 40 45

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
50 55 60

Glu Gln Gln Ala Leu Gln Thr Val Gly Ser Gln Ile Phe Val Lys Thr
65 70 75 80

Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
85 90 95

Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp
100 105 110

Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr
115 120 125

Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu
130 135 140

Arg Leu Arg Gly Gly Ser
145 150

<210> 28
<211> 338
<212> PRT

<213> Artificial

<220>
<223> H6-IEPD-RAP

<400> 28

Met Gly Ser His His His His His His Gly Ser Ile Glu Pro Asp Tyr
1 5 10 15

Ser Arg Glu Lys Asn Gln Pro Lys Pro Ser Pro Lys Arg Glu Ser Gly
20 25 30

Glu Glu Phe Arg Met Glu Lys Leu Asn Gln Leu Trp Glu Lys Ala Gln
35 40 45

Arg Leu His Leu Pro Pro Val Arg Leu Ala Glu Leu His Ala Asp Leu
50 55 60

Lys Ile Gln Glu Arg Asp Glu Leu Ala Trp Lys Lys Leu Lys Leu Asp
65 70 75 80

Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn Leu
85 90 95

Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala Arg
100 105 110

Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu Asp
115 120 125

Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys
130 135 140

Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His
145 150 155 160

Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg
165 170 175

Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp
180 185 190

Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys
195 200 205

Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His
210 215 220

Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp
225 230 235 240

Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu
245 250 255

Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His
260 265 270

Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His
275 280 285

Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys
290 295 300

His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys
305 310 315 320

Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn
325 330 335

Glu Leu

<210> 29
<211> 197
<212> PRT
<213> Artificial

<220>
<223> H6-IEPD-TN123

<400> 29

Met Gly Ser His His His His His His Gly Ser Ile Glu Pro Asp Gly
1 5 10 15

Glu Pro Pro Thr Gln Lys Pro Lys Lys Ile Val Asn Ala Lys Lys Asp
20 25 30

Val Val Asn Thr Lys Met Phe Glu Glu Leu Lys Ser Arg Leu Asp Thr
35 40 45

Leu Ala Gln Glu Val Ala Leu Leu Lys Glu Gln Gln Ala Leu Gln Thr
50 55 60

Val Cys Leu Lys Gly Thr Lys Val His Met Lys Cys Phe Leu Ala Phe
65 70 75 80

Thr Gln Thr Lys Thr Phe His Glu Ala Ser Glu Asp Cys Ile Ser Arg
85 90 95

Gly Gly Thr Leu Ser Thr Pro Gln Thr Gly Ser Glu Asn Asp Ala Leu
100 105 110

Tyr Glu Tyr Leu Arg Gln Ser Val Gly Asn Glu Ala Glu Ile Trp Leu
115 120 125

Gly Leu Asn Asp Met Ala Ala Glu Gly Thr Trp Val Asp Met Thr Gly
9

130 135 140

Ala Arg Ile Ala Tyr Lys Asn Trp Glu Thr Glu Ile Thr Ala Gln Pro
145 150 155 160

Asp Gly Gly Lys Thr Glu Asn Cys Ala Val Leu Ser Gly Ala Ala Asn
165 170 175

Gly Lys Trp Phe Asp Lys Arg Cys Arg Asp Gln Leu Pro Tyr Ile Cys
180 185 190

Gln Phe Gly Ile Val
195

<210> 30
<211> 150
<212> PRT
<213> Artificial

<220>
<223> H6-TripUB IQADISP

<400> 30

Met Gly Ser His His His His His His Gly Ser Gly Ser Gly Ser Ile
1 5 10 15

Gln Ala Asp Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys
20 25 30

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
35 40 45

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
50 55 60

Glu Gln Gln Ala Leu Gln Thr Val Gly Ser Gln Ile Phe Val Lys Thr
65 70 75 80

Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
85 90 95

Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp
100 105 110

Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr
115 120 125

Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu
130 135 140

Arg Leu Arg Gly Gly Ser
145 150

<210> 31
<211> 150
<212> PRT
<213> Artificial

<220>
<223> H6-TripUB IQAD!SG

<400> 31

Met Gly Ser His His His His His His Gly Ser Gly Ser Gly Ser Ile
1 5 10 15

Gln Ala Asp Ser Gly Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys
20 25 30

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
35 40 45

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
50 55 60

Glu Gln Gln Ala Leu Gln Thr Val Gly Ser Gln Ile Phe Val Lys Thr
65 70 75 80

Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
85 90 95

Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp
100 105 110

Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr
115 120 125

Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu
130 135 140

Arg Leu Arg Gly Gly Ser
145 150

<210> 32
<211> 150
<212> PRT
<213> Artificial

<220>
<223> H6-TripUB VGPD!SP

<400> 32

Met Gly Ser His His His His His His Gly Ser Gly Ser Gly Ser Val
1 5 10 15

Gly Pro Asp Ser Pro Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys
20 25 30

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
11

35 40 45

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
50 55 60

Glu Gln Gln Ala Leu Gln Thr Val Gly Ser Gln Ile Phe Val Lys Thr
65 70 75 80

Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
85 90 95

Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp
100 105 110

Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr
115 120 125

Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu
130 135 140

Arg Leu Arg Gly Gly Ser
145 150

<210> 33
<211> 150
<212> PRT
<213> Artificial

<220>
<223> H6-TripUB VGPD!FG

<400> 33

Met Gly Ser His His His His His His Gly Ser Gly Ser Gly Ser Val
1 5 10 15

Gly Pro Asp Phe Gly Gly Thr Glu Pro Pro Thr Gln Lys Pro Lys Lys
20 25 30

Ile Val Asn Ala Lys Lys Asp Val Val Asn Thr Lys Met Phe Glu Glu
35 40 45

Leu Lys Ser Arg Leu Asp Thr Leu Ala Gln Glu Val Ala Leu Leu Lys
50 55 60

Glu Gln Gln Ala Leu Gln Thr Val Gly Ser Gln Ile Phe Val Lys Thr
65 70 75 80

Leu Thr Gly Lys Thr Ile Thr Leu Glu Val Glu Pro Ser Asp Thr Ile
85 90 95

Glu Asn Val Lys Ala Lys Ile Gln Asp Lys Glu Gly Ile Pro Pro Asp
100 105 110

Gln Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu Glu Asp Gly Arg Thr
115 120 125

Leu Ser Asp Tyr Asn Ile Gln Lys Glu Ser Thr Leu His Leu Val Leu
130 135 140

Arg Leu Arg Gly Gly Ser
145 150